PRIMATE VENTROMEDIAL PREFRONTAL CORTEX AND THE PHYSIOLOGICAL AND BEHAVIOURAL DYSFUNCTION CHARACTERISTIC OF MOOD AND ANXIETY DISORDERS

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ABSTRACT

Title: Primate Ventromedial Prefrontal Cortex and the Physiological and Behavioural Dysfunction Characteristic of Mood and Anxiety Disorders

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The heterogeneity intrinsic to the ventromedial prefrontal cortex (vmPFC) is evidenced in both its anatomy and implicated function: vmPFC subregions have roles in positive affect, negative affect and autonomic/endocrine regulation. Whether different subregions serve fundamentally different functions, or whether they perform similar computations on different inputs, remains unclear. Nevertheless, the role of the vmPFC in psychopathology is widely appreciated – in mood and anxiety disorders, over-activity within constituent regions of the vmPFC is consistently implicated in symptomatology, together with its normalisation following successful treatment. However, the precise locus of change varies between studies.

The work presented in this thesis investigates the causal contributions of over-activity within two key subregions of the vmPFC – the subgenual anterior cingulate cortex (sgACC, area 25) and perigenual anterior cingulate cortex (pgACC, area 32) – in discrete dimensions of behaviour and physiology affected in psychiatric disorders. Specifically, the impact of over-activity is assessed on (i) baseline physiological function; (ii) the regulation of anticipatory, motivational and consummatory aspects of reward-related behaviour; and (iii) negative affect including fear learning, stress recovery and the intolerance of uncertainty. To provide further insight into the mechanism of action of antidepressants, the efficacy of selected treatments is tested on changes induced by over-activity of these regions.

Beyond the direct relevance of the results presented here to psychiatric disorders and their treatment, the thesis aims to emphasise the importance of broader themes associated with the measurement and quantification of emotion in preclinical animal studies. First, a multi-faceted approach is utilised enabling quantification of both the autonomic and behavioural aspects of emotion. In so doing, the experiments maintain relevance to studies which assess these correlates in isolation, both in humans (which typically measure subjective responses and physiology) and in rodents (which frequently assess behaviour in isolation). The assessment of more than one dimension of emotion confers these studies with improved power to detect maladaptive changes. Second, the experiments described were conducted in the marmoset, a new-world primate. The extensive anatomical homology between marmoset and human prefrontal cortex facilitates the forward-translation of functional results. In combination with the appropriate assays, this renders marmosets as an invaluable species to study the causal contributions of vmPFC subregions to symptoms of psychiatric disorders.

I believe that the results of these experiments provide important insights into the causal role primate vmPFC has in relation to the behavioural and physiological aspects of psychiatric symptomatology. Most importantly, I hope that they serve as the foundation for future work to further elucidate the neuropathological processes underlying mental disorders.

To Ragheb, Joanne and Anna

PREFACE

The following work was carried out at the Department of Physiology, Development and Neuroscience, University of Cambridge, during the years 2015-2018, under the supervision of Professor Angela C. Roberts.

The dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

The dissertation does not exceed the prescribed word limit of 80,000 for the Degree Committee of Biology.

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Publications

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I am indebted to every single person I have worked with at the Innes, without whom these experiments would simply not have been possible. I mean this not only from a practical standpoint – thank you for holding my animals – but also because of their support as friends. I must particularly thank Dr Philip Gaskin for his work with the touchscreen and sucrose preference animals, Dr Nicole Horst for her guidance during some of the most complicated experiments carried out as part of this thesis, Dr Roohollah Massoudi for his help in setting up the telemetry system (and some great debates in the Yellow Room), Lauren Anderson for her tireless efforts (and patience!) helping me with scoring and animal-holding, and Gemma Cockcroft for histological processing and immunohistochemistry.

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Finally, to my parents, sister and grandparents: your unconditional and unwavering support has allowed me to grow and flourish into the man that I am today, and for that I am eternally grateful. I love you.

ABBREVIATIONS

The following abbreviations are used in this thesis:

Abbreviation	Meaning
^{18}F-FDG PET	^{18}Fluorine-fluorodeoxyglucose positron emission tomography
5,7DHT	5,7-dihydroxytryptamine
5HIAA	5-hydroxyindoleacetic acid
5HT	Serotonin
5HTT	Serotonin reuptake transporter
5HTTLPR	Serotonin reuptake transporter long promoter region
6OHDA	6-hydroxydopamine
AAV	Adeno-associated virus
AC	Anterior cingulate region (of rodent mPFC)
ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
AMPA	*α*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AP	Anteroposterior
aPFC	Anterior prefrontal cortex
BA	Brodmann Area
BAS	Behavioural activation system
BDNF	Brain-derived neurotrophic factor
BIS	Behavioural inhibition system
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
BOLD	Blood oxygen level dependent
BP	Blood pressure
BrkP	Breakpoint
CaMKIIa	Calcium/calmodulin dependent protein kinase promoter (DREADDs)
CAN	Central autonomic network
CBT	Cognitive-behavioural therapy
CeN	Central nucleus
CGP/LY	CGP52432/ LY341495
CGT	Cambridge Gambling Task
CMS	Chronic mild stress
CNO	Clozapine-*N*-oxide
CNS	Central nervous system
Cort(Num)	Cortisol (dose in mg/kg)
CPAS	Chapman Physical Anhedonia Scale
CR	Conditioned response
CRH	Corticotropin releasing hormone
CS	Conditioned stimulus
CSAS	Chapman Social Anhedonia Scale
CSF	Cerebrospinal fluid

CSI	Cardiac sympathetic index
CSPT	Cortico-striato-pallido-thalamic
CVI	Cardiac vagal index
DA	Dopamine
DAB	Diaminobenzidine
dACC	Dorsal anterior cingulate cortex
DBS	Deep brain stimulation
DHK	Dihydrokainic acid
dlPFC	Dorsolateral prefrontal cortex
DMH	Dorsomedial hypothalamus
DMN	Default mode network
dmPFC	Dorsomedial prefrontal cortex
DREADD	Designer receptor exclusively activated by designer drug
DRN	Dorsal raphe nucleus
DSM	Diagnostic and Statistical Manual of Mental Disorders
EAAT2	Excitatory amino acid transporter-2
ECT	Electroconvulsive therapy
eEF2	Eurkaryotic elongation factor 2
EEfRT	Effort Expenditure for Reward Task
EEG	Electroencephalography
EFA	Exploratory factor analysis
EPN	Emotional processing network
ET	Endotracheal
F	F statistic: ratio of MS_{effect} to MS_{error}
FCPS	Fawcett-Clark Pleasure Scale
fMRI	Functional Magnetic Resonance Imaging
FR	Fixed ratio
FRP	Facial reactivity pattern
GABA	*γ*-aminobutyric acid
GAD	Generalised anxiety disorder
GCR	Glucocorticoid receptor
Glx	Combined glutamate and glutamine concentration (MRS)
GPCR	G-protein coupled receptor
GWAS	Genome-wide association study
HA	Haemagglutinin (DREADDs)
HARS	Hamilton Anxiety Rating Scale
HDRS	Hamilton Depression Rating Scale
HI	Human intruder
$hM_{3/4}D_{q/i}$	Protein-engineered muscarinic receptor (DREADDs)
HPA	Hypothalamo-pituitary-adrenal
HR	Heart rate
HRV	Heart rate variability
hSyn	Human synapsin promoter (DREADDs)
IBI	Inter-beat interval
ICD	International Classification of Diseases
ICSS	Intracranial self-stimulation
IGT	Iowa Gambling Task

IL	Infralimbic (cortex)
IML	Intermediolateral (nucleus of the thoracic spinal cord)
IRES	Internal ribosomal entry site (DREADDs)
ITI	Inter-trial interval
KMO	Kaiser-Meyer-Olkin
LC	Locus coeruleus
LH	Lateral hypothalamus
LM	Lateromedial
lOFC	Lateral orbitofrontal cortex
lPFC	Lateral prefrontal cortex
LSD	Least squares difference
LTD	Long term depression
LTP	Long term potentiation
MAOi	Monoamine oxidase inhibitor
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
mCitrine	Fluorescent tag (DREADDs)
MCR	Mineralocorticoid receptor
MD	Mediodorsal nucleus (of the thalamus)
MDD	Major depressive disorder
MI	Myocardial infarction
MID	Monetary incentive delay
MO	Medial orbital (region of rodent mPFC)
mOFC	Medial orbitofrontal cortex
mPFC	Medial prefrontal cortex
MRF	Medullary reticular formation
MRI	Magnetic resonance imaging
MRN	Median raphe nucleus
MRS	Magnetic resonance spectroscopy
MST	Magnetic seizure therapy
NA	Noradrenaline/noradrenergic
NHP	Non-human primate
NMDA	*N*-methyl-D-aspartate (receptor)
NS	Not significant
NTS	Nucleus tractus solitarius (nucleus of the solitary tract)
NVI	Neurovisceral integration
OCD	Obsessive-compulsive disorder
OFC	Orbitofrontal cortex
oPFC	Orbital prefrontal cortex
PAG	Periaqueductal gray
PET	Positron Emission Tomography
PFC	Prefrontal cortex
pgACC	Perigenual anterior cingulate cortex
PIT	Pavlovian-to-instrumental transfer
PL	Prelimbic (cortex)
PLv	Ventral prelimbic (cortex)
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus (of the hypothalamus)

RDoC	Research Domain Criteria
RMSSD	Root mean squared standard deviation
RNA	Ribonucleic acid
RSC	Retrosplenial cortex
rTMS	Repetitive transcranial magnetic stimulation
rvmPFC	Rostral ventromedial prefrontal cortex
SAD	Social anxiety disorder
SC	Subcutaneous
SEM	Standard error of the mean
sgACC	Subgenual anterior cingulate cortex
SHaPS	Snaith-Hamilton Pleasure Scale
siRNA	Short inhibitory ribonucleic acid
SNRI	Serotonin-noradrenaline reuptake inhibitor
SSRE	Selective serotonin reuptake enhancer
SSRI	Selective serotonin reuptake inhibitor
SUVR(c)	Standard uptake value ratio (normalised to cerebellum)
TCA	Tricyclic antidepressant
tDCS	Transcranial direct current stimulation
TEPS	Temporal Experience of Pleasure Scale
TEPS-ANT	Anticipatory TEPS scale
TEPS-CONS	Consummatory TEPS scale
TSAB	Time spent at back
TSAF	Time spent at front
US	Unconditioned stimulus
VMAT	Vesicular monoamine transporter
vmPFC	Ventromedial prefrontal cortex
VNS	Vagal nerve stimulation
VS	Vigilant scanning
VTA	Ventral tegmental area
α	Threshold for determining statistical significance`

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1 General Introduction

Neuropsychiatric illnesses are common and debilitating, and of these, depression and anxiety are associated with the largest disease burden (Whiteford et al., 2013). Both preclinical interventional studies in animals and correlative neuroimaging studies in humans have provided significant insights into the wide-spread neuroanatomical, neurophysiological and neurochemical abnormalities associated with specific symptoms. Many of these disorders share prefrontal cortex (PFC) dysfunction as an important neural signature (Godsil et al., 2013; Myers-Schulz and Koenigs, 2012; Price and Drevets, 2010). Perhaps unsurprisingly given its role in the regulation of emotion, changes in activity within the ventromedial PFC (vmPFC) – including the subgenual anterior cingulate cortex (sgACC) and perigenual anterior cingulate cortex (pgACC) – have been repeatedly identified in the context of mood and anxiety disorders. However, the precise locus of change varies from study-to-study, and forward translation from preclinical studies is made difficult owing to a lack of understanding regarding the functional equivalence of sectors of rodent vmPFC to those of primates. These difficulties are further compounded by inconsistent terminology and imprecise definitions concerning the anatomy of the vmPFC, and the PFC more generally.

In this chapter, literature concerning the anatomy of the PFC and vmPFC will be discussed first, to provide an anatomical framework within which functional results can be interpreted. Then, the role of the vmPFC in the regulation of appetitive and aversive behaviour will be considered, together with its role in autonomic and endocrine function – and whether these diverse functions can be parcellated at a neuroanatomical level. Finally, the vmPFC will be discussed in the context of psychiatric disorders, with a focus on mood disorders – primarily depression – and anxiety disorders.

1.1 Defining the Prefrontal Cortex and the Ventromedial Prefrontal Cortex

1.1.1 Defining the prefrontal cortex

The PFC is loosely defined as the portion of the frontal lobe anterior to the premotor and primary motor cortex. Consensus on the precise neuroanatomical constituents of the PFC has yet to be reached, although there are several proposals:

- **Region of frontal cortex which, when stimulated, does not lead to observable movements** – This definition was adopted by Ferrier in the late 19th century (Ferrier, 1890) and was one of the earliest definitions of the PFC. However this fell out of use with the development of cytoarchitectonic definitions (see below), following the

recognition that electrically silent frontal cortex encompasses highly heterogeneous cytoarchitectonic regions (Preuss, 1995).

- **Region of frontal cortex which receives projections from the mediodorsal (MD) nucleus of the thalamus** – Formulated originally by Rose and Woolsey (Rose and Woolsey, 1948) and developed further by Akert (Akert, 1964), this definition is often used in the context of defining cross-species homologues: regions of frontal cortex receive MD projections across a number of species, including primates and rodents. This definition is used to this day, although it is limited by the fact that MD thalamus projections are relatively widespread and not restricted to definitive group of cytoarchitectonic regions in primates (Preuss, 1995). This definition has since been modified to suggest that the PFC has stronger *reciprocal* connections to MD thalamus compared with any other thalamic nucleus (Uylings et al., 2003).
- **Dopaminergic projections from the midbrain** – In the 1970s, catecholaminergic innervation of the frontal cortex of macaques was being extensively studied. Preliminary accounts of the distribution of dopaminergic fibres gave rise to the suggestion that primate PFC is specifically innervated by the dopaminergic nuclei of the midbrain (Björklund et al., 1978; MacBrown and Goldman, 1977). Indeed, Divac and colleagues demonstrated that the distribution of dopaminergic terminations is coextensive with MD projections in primates and non-primates (Divac et al., 1978), thereby showing cross-validity with another approach for defining the PFC. However, dopaminergic terminations have also been identified in mid ACC, premotor cortex and primary motor cortex (Berger et al., 1991; Gaspar et al., 1992). Although these are regions of frontal cortex, they are typically considered too caudal to constitute PFC and are functionally very distinct.
- **Cytoarchitectonic definitions** – The anatomist Korbinian Brodmann suggested that PFC was defined by the presence of small, granule cells in layer II and layer IV (Brodmann, 1909). In his initial investigations, he observed two different patterns of layer IV granularity in the frontal lobe – no granule cell layer (agranular), or a thick, well defined granule cell layer (granular). Based on this, he grouped frontal lobe regions into an agranular precentral region (*regio praecentralis*), an agranular medial region (*regio cingularis*) and a granular frontal region (*regio frontalis*; including dorsal, lateral and ventral surfaces of the frontal lobe). The *regio frontalis* was generally and collectively referred to as the PFC until the mid-20th century (Groenewegen et al., 1997) and it was the presence of a distinct granular layer IV that was the key feature distinguishing *regio frontalis* (PFC) from the agranular premotor and primary motor cortex. The ACC – including dorsal (d), perigenual (pg) and subgenual (sg) regions – was considered separate as it was agranular. Brodmann further asserted that

granular frontal cortex is unique to primates, since other species he studied did not have a granular layer in cortex of the frontal lobe. Indeed, according to Brodmann's definition, rodents do not possess a PFC as their cortex completely lacks a granular layer IV (Uylings et al., 2003).

Since his initial work, subsequent studies have shown that several of the areas identified by Brodmann as granular do not have a clear layer IV – instead, it is a sparse and thinly developed. Anatomists refer to these areas as being 'dysgranular,' and include areas on the medial wall and ventral surface of the frontal lobe (Barbas and Pandya, 1989; Walker, 1940). In addition, owing to its extensive connectivity with the MD thalamus, the separation between ACC and PFC has become blurred, and most investigators consider rostral portions of the ACC as being part of the PFC (Barbas, 2015). Despite these discrepancies, Brodmann's characterisation of the PFC has been enormously useful because it can be used to fractionate the PFC into subregions based on relatively well-defined cytoarchitectonic characteristics (see below).

In rodents and primates, the PFC is appreciated to be a heterogeneous brain region (**FIGURE 1-1**) and attempts have been made to delineate subregions of the PFC in a manner that is comparable across species. However, language relating to these subregions is often imprecise: whilst they can be useful, terms including 'medial' PFC (mPFC), 'orbital' PFC (oPFC, also termed orbitofrontal cortex, OFC), 'anterior' PFC (aPFC), 'lateral' PFC (lPFC) and vmPFC are used when discussing the PFC without detailed characterisation of the brain regions to which they refer. Brodmann's work gave rise to the earliest iteration of a series of comparative maps of the cytoarchitectonic subregions of human and non-human primate (NHP) frontal cortex (Brodmann, 1909). These subregions were numbered – for example, in the macaque PFC, *regio frontalis* was comprised of BA10, 11, 12 and 13 ventrally and BA8 and 9 laterally. *Regio cingularis* (then not considered part of the PFC) was comprised of agranular BA24, 25 and 32.

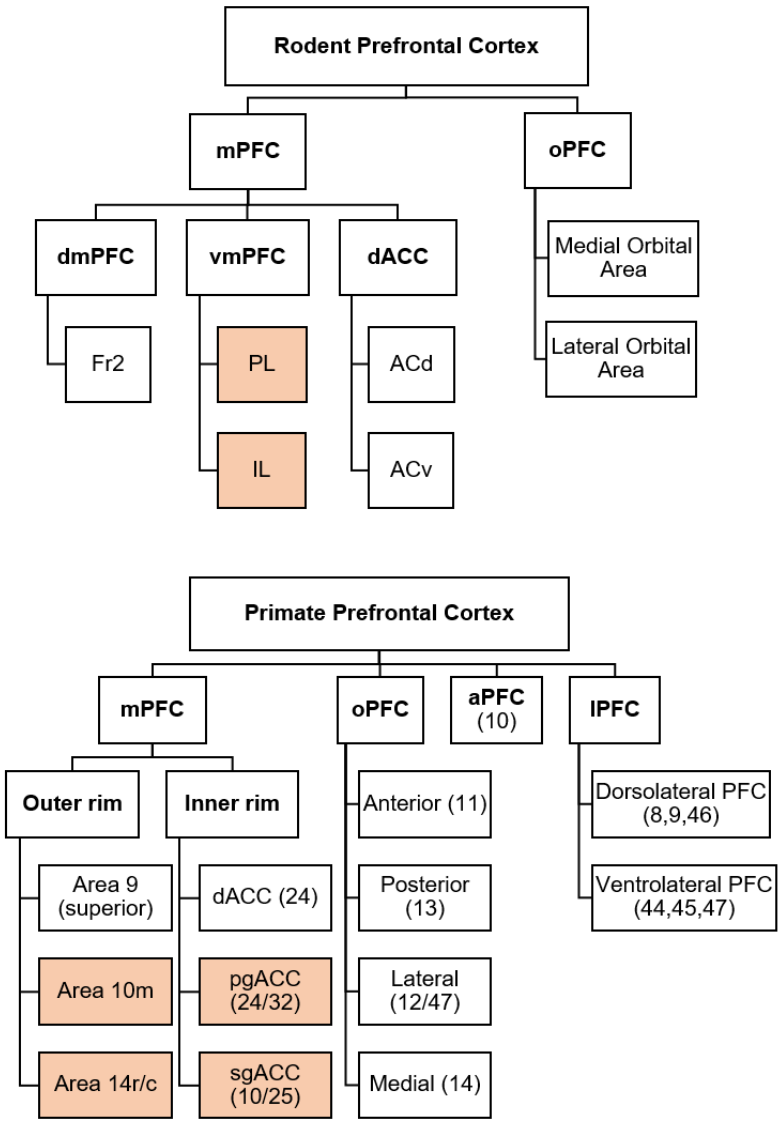


Figure 1-1 In both rodents and primates, the PFC is a heterogeneous brain region.
Subregions typically classified as contributing to 'ventromedial' PFC (vmPFC) are highlighted in red. **A** The rodent PFC is subdivided into medial and orbital zones. Rodents do not have a lateral prefrontal cortex – instead, executive function is thought to be subserved by dorsomedial PFC (dmPFC). Rodent vmPFC typically refers to prelimbic (PL) and infralimbic (IL) sectors, but variably includes the anterior cingulate ventral (ACv) division. **B** Primate PFC is more extensive, consisting of loosely defined mPFC, oPFC, anterior PFC (aPFC, sometimes termed frontopolar cortex) and lateral PFC (lPFC). The mPFC of primates can be thought of as comprising a superficial 'outer rim' and a deep 'inner rim' hugging the corpus callosum. In primates, references to the vmPFC typically include perigenual anterior cingulate cortex (pgACC, including BA24 and BA32), subgenual anterior cingulate cortex (including caudally, BA25 and rostrally, BA10), BA10m (sometimes referred to as rostral 'r'vmPFC) and BA14 (both rostral – 14r – and caudal – 14c – divisions).

The focus of this thesis is the role of PFC subregions in the regulation of emotion, and the subdivision of the PFC most consistently implicated in emotion and its regulation is the

vmPFC (Myers-Schulz and Koenigs, 2012). Therefore, subsequent discussion of the anatomy of the PFC and its role in (ab)normal emotion will focus on the vmPFC. As has been mentioned, discussion is invariably constrained by anatomical imprecision, but wherever possible the precise subregions to which studies refer will be quoted.

1.1.2 Defining the ventromedial prefrontal cortex

As with other subregions, the term vmPFC is often used loosely, without a precise characterisation of the brain regions to which it refers. In this thesis, the term vmPFC will refer to the subregions outlined in **FIGURE 1-1**.

1.1.2.1 Rodent ventromedial prefrontal cortex

Brodmann did not consider the rat to have a PFC (and, by extension, a vmPFC) because its frontal cortex is entirely agranular (Brodmann, 1909). Defining rodent prefrontal and ventromedial prefrontal subregions remains difficult for this very reason, although subsequent investigators have asserted that the rat does possess prefrontal regions homologous to the oPFC, mPFC and vmPFC of primates. These anatomists have tended to adopt a characterisation based on projections of the MD nucleus of the thalamus (see **1.1.1**). The first example of this was the work of Krettek and Price, who traced efferent projections of the MD thalamus and characterized four subdivisions of the rodent mPFC: prelimbic (PL), infralimbic (IL), anterior cingulate (AC, dorsal (d) and ventral (v) subfields) and medial orbital (MO) (Krettek and Price, 1977) (**FIGURE 1-2A**). Together, the PL and IL regions were said to constitute rodent vmPFC. Following on from Krettek and Price's work assessing MD projection zones (and with the growing consensus that 'PFC' included agranular regions), Vogt and Peters evaluated the cytoarchitecture of the rat cingulate cortex in light of Brodmann's description using Golgi staining (Vogt and Peters, 1981). Vogt and Peters emphasized the homology between subregions of rodent vmPFC and primate vmPFC by adopting Brodmann's nomenclature: PL corresponded to BA32, IL corresponded to BA25 and AC corresponded to BA24 (**FIGURE 1-2B**). This equivalence has become widely adopted when discussing both rodent-monkey (Gabbott et al., 2003) and rodent-human (Quirk and Beer, 2006) homology. Indeed, subsequent work has shown that beyond having similar cytoarchitecture and locations with the PFC, PL/BA32 and IL/BA25 have similar afferent/efferent connectivity (Haber, 2016; Passingham and Steven, 2012; Price, 2007).

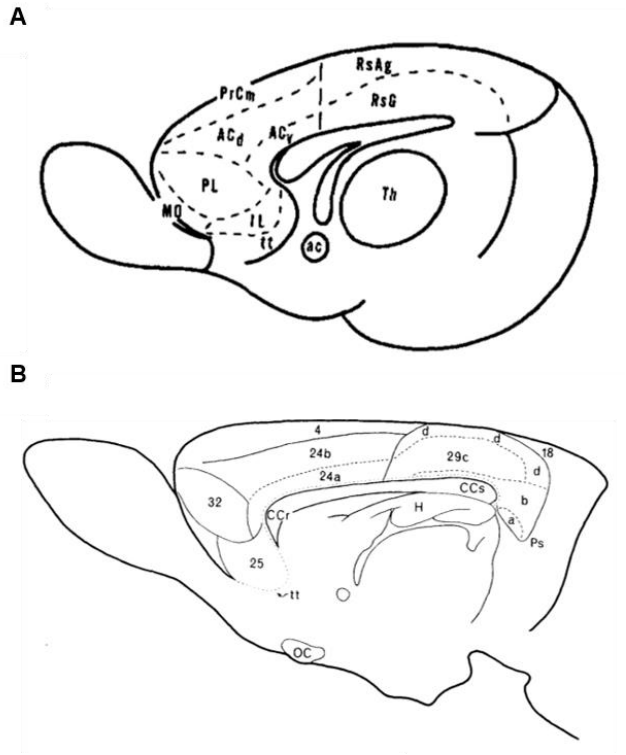


Figure 1-2 Medial wall of the rodent brain. **A** Krettek and Price (1977) fractionated rodent cortex based on projections of the MD thalamus and identified four subregions constituting mPFC – infralimbic (IL), prelimbic (PL), anterior cingulate (AC, dorsal (d) and ventral (v)) and medial orbital (MO). **B** Vogt and Peters (1981) identified similar anterior divisions to Krettek and Price but used different nomenclature. 'Area 25' is roughly equivalent to IL; 'area 32' to PL; '24a' to ACv; and '24b' to ACd. MO was not described in this work, although an analogous region is bordered by 'area 25/32'.

1.1.2.2 Non-human primate ventromedial prefrontal cortex

Classification systems for NHP vmPFC have developed extensively over the 20th and 21st centuries (Schmahmann and Pandya, 1997). Brodmann characterized the cellular architecture of the PFC in rhesus macaque monkeys (**FIGURE 1-3A**), applying his numbering scheme to specific subregions. However, there were differences in his characterisation of subregions within NHP vmPFC compared to those identified in humans – specifically, there was difficulty with BA32, and Brodmann stated that his 'monkey' BA32 was not homologous to human BA32.

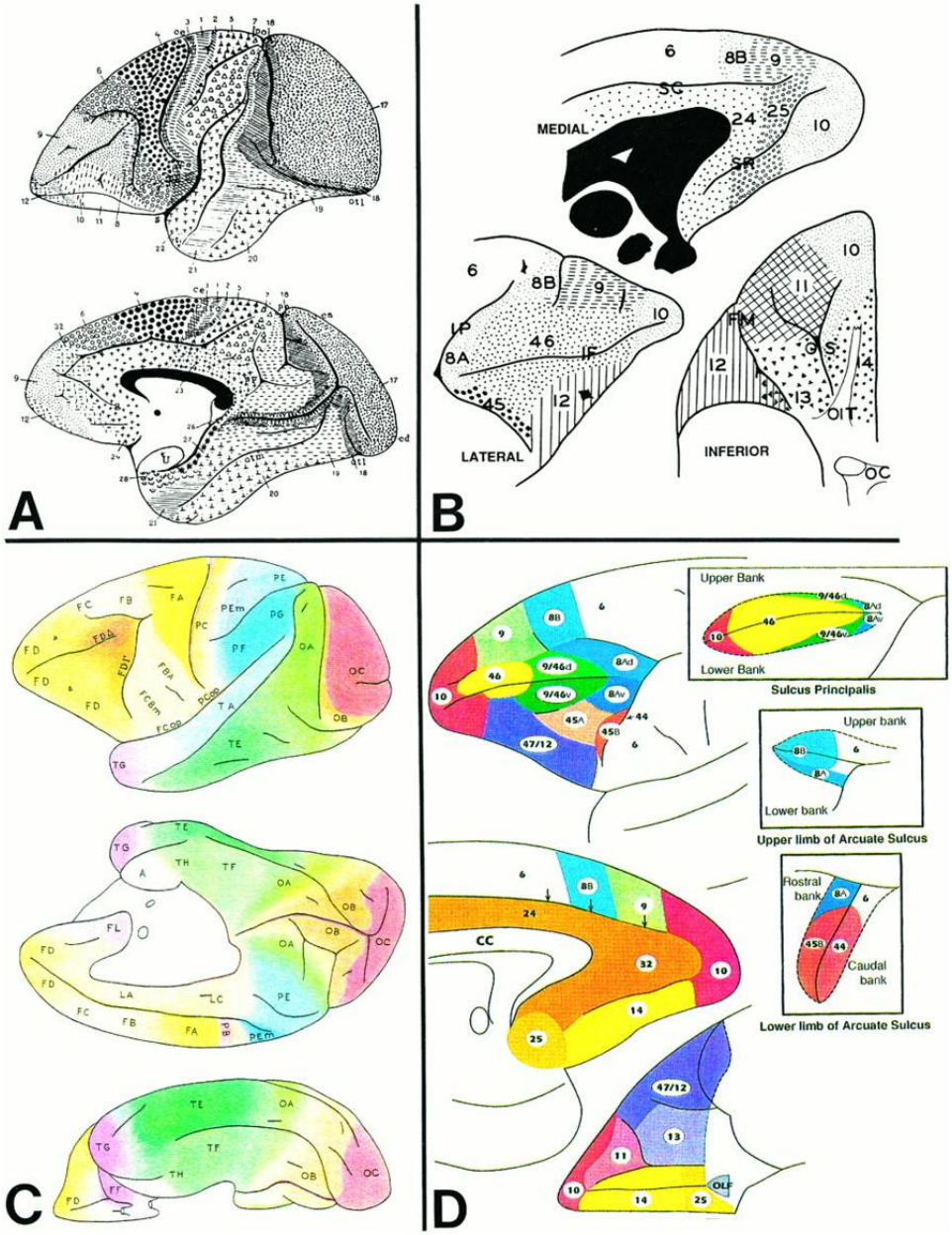


***Figure 1-3 Different maps illustrating cytoarchitectonic areas of the cerebral cortex of the rhesus macaque*.** Taken from Schmahmann and Pandya, 1997. In all cases, the PFC is recognised as a heterogenous brain region that covers an extensive portion of the macaque frontal lobe. **A** Designation of Brodmann (1909). vmPFC consists of BA24 and 32. **B** Designation of Walker (1940). vmPFC consists of BA24 and 25. **C** Designation of von Bonin and Bailey (1947). Note difference in nomenclature. vmPFC consists of 'FL' (corresponding to BA25) and 'FD' (corresponding to BA32). **D** Designation of Petrides and Pandya (1994). vmPFC consists of BA25, 32 and 14.

Differences between the anatomical characterisation of macaque and human PFC led Walker (1940) to re-examine Brodmann's maps. Walker realised that several BAs could be further sub-divided based on further examination of their cytoarchitectonic features. In

Walker's first revised map, there was closer correspondence to the human PFC (**FIGURE 1-3B**). Even in Walker's map, BA32 was not recognized at all. Instead, BA24 and 25 constituted the entirety of the vmPFC (Walker, 1940). At a similar time, von Bonin and Bailey also published a revised map of macaque neocortex (**FIGURE 1-3C**) (von Bonin and Bailey, 1947), using lettering terminology on von Economo's parcellation of human cortex (see **1.1.2.3**). Whilst the lettering system adopted by von Bonin and Bailey failed to become popular, von Bonin and Bailey's map was very similar to that of Walker's.

The Walker map remained a mainstay until the 1980s, when more sophisticated staining techniques were used to identify architectonic features not possible to delineate previously. Vogt and colleagues first identified BA32 in the macaque, comprised of thick layers II-IV and distinct 'band' in layer V (Vogt et al., 1987). Barbas and Pandya carried out further work fractionating macaque PFC, distinguishing BA25, BA32 and BA24 on the basis of cyto- and also *myeloarchitectural* differences: for example, BA32 has a more discernible cortical layer II (cytoarchitectural) and a faint inner Baillarger band (myelinated fibres travelling from layer V; myeloarchitectural) (Barbas and Pandya, 1989). These findings were in broad agreement with the earlier cytoarchitectonic work from Vogt.

In the 1990s, the focus was extended from cyto- and myeloarchitecture to encompass consideration of *chemoarchitectural* differences. Carmichael and Price conducted an extended analysis of subregions of NHP PFC in three different macaque species (Carmichael and Price, 1994). In addition to cytoarchitectural and myeloarchitectural differences, Carmichael and Price considered markers related to metabolism, synapses and neurotransmission. In this work, more than 20 different fields were identified in orbital, medial and ventromedial regions. BA24, BA25 and BA32 were delineated on the medial wall, separated from surrounding PFC by dark acetylcholinesterase (AChE) staining. AChE could also be used to separate these regions (albeit with low resolution), with BA25 containing the most AChE+ fibres. Calbindin separated BA25, BA32 and BA24 more distinctly – whilst BA24 had many calbindin+ cell bodies, in BA25 calbindin+ cell bodies were scattered across all cortical layers and in BA32 staining was especially sparse. However, it is worth noting that the border between BA25 and BA32 was gradual – a low degree of precision was achieved when attempting to identify the cortical boundaries. In parallel, Petrides and Pandya developed their own map of macaque PFC based on cytoarchitectural and *connectional* characteristics (**FIGURE 1-3D**) (Petrides and Pandya, 1994). To define the connectivity of prefrontal subregions, Petrides and Pandya microinjected anterograde and retrograde tracers, to determine the efferent and afferent connectivity respectively. Based on these properties, the medial wall of the Petrides-Pandya map includes BA24, BA25 and a

particularly extensive BA32 occupying the majority of the vmPFC. Ventral to BA32, a medial component of BA14 extends around onto the inferior aspect of the medial wall.

Most recently, there has been a push for *quantitative architectonics* using a multi-dimensional approach with a combination of techniques. This approach has been pioneered by the work of Helen Barbas and colleagues (for example, in (Dombrowski et al., 2001)). Using fundamental architectonic criteria – including cyto-, myelo- and chemoarchitectonic features – Barbas and colleagues have investigated whether subregions of the PFC have unique profiles that can be illustrated quantitatively. Dombrowski *et al*. have shown that neuronal density is highly informative when establishing architectonic profiles in the macaque, followed by measures of cortical thickness and parvalbumin+ neuron density. Interestingly, the use of this approach led to BA25 being characterised as an 'outlier' region, distinct from its neighbouring vmPFC subregions, based on quantitative analysis of these architectonic features.

The characterisation of NHP BA25 is worth further consideration, because the borders of BA25 are subject to variability across different maps. Petrides and Pandya (1994), Dombrowski *et al*. (2001) and other work by Helen Barbas (e.g. Barbas and Pandya, 1989) identifies a portion of BA25 that extends ventrally onto the orbital surface, abutting BA13, in addition to the 'typical' portion BA25 on the medial wall. By contrast, Carmichael and Price label an analogous region to Barbas *et al.*'s orbital BA25 as BA14c (**FIGURE 1-4**). In their paper Carmichael and Price show that BA14c differs markedly to BA14r based on PV neuron distribution and AChE staining (see (Carmichael and Price, 1994) Figure 8 for PV; Figure 20A,C for AChE), and based on data from their own manuscript it would appear that 14c is actually more similar to BA25. Therefore, in the macaque, it is likely that BA25 extends from the medial surface onto the orbital surface.

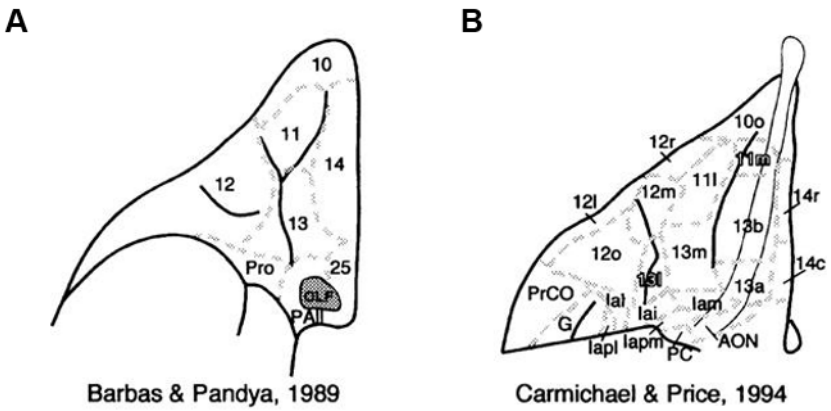


Figure 1-4 Comparison of the orbital surface of the rhesus macaque PFC as fractionated by Barbas and Pandya (1989) vs. Carmichael and Price (1994). **A** In the map by Barbas and

Pandya, an orbital portion of BA25 is identified based on chemo-architectural and quantitative architectonic differences. **B** Carmichael and Price identify a caudal region of BA14(c), which may be equivalent to orbital BA25.

Beyond these extensive characterisations of macaque vmPFC (an old-world monkey), the neuroanatomy of marmoset vmPFC (a new-world monkey) has also been investigated. Knowledge of marmoset neuroanatomy is proving increasingly important, as the marmoset is becoming a popular experimental system for neuroscience research (Oikonomidis et al., 2016). Remarkably, in his original work, Brodmann included a cytoarchitectonic map of marmoset cortex (then genus *Hapale*, now *Callithrix*) in his cross-species comparisons of cytoarchitecture (**FIGURE 1-5**). In his analysis, marmosets and macaques had equivalent regions of the PFC: the marmoset vmPFC contained BA25, 24 and 32. Characterisation of the marmoset vmPFC was not carried out in much further detail until 2009, when Burman and Rosa used a combination of cyto-, myelo- and chemoarchitectural (cytochrome oxidase) approaches to identify subregions of marmoset orbital and medial PFC which were likely homologous to those seen in Old World monkeys (Burman and Rosa, 2009). From their work, it is apparent that many of the subregions found in Old World monkey vmPFC can be found in the marmoset, including BA25, BA32 and BA24a/b. In addition to being evolutionarily informative (suggestive of a basic underlying organisation of primate frontal cortex), knowledge of the subdivisions of marmoset vmPFC provides an essential anatomical framework for interpreting functional studies in the marmoset (including all the experimental work in this thesis).

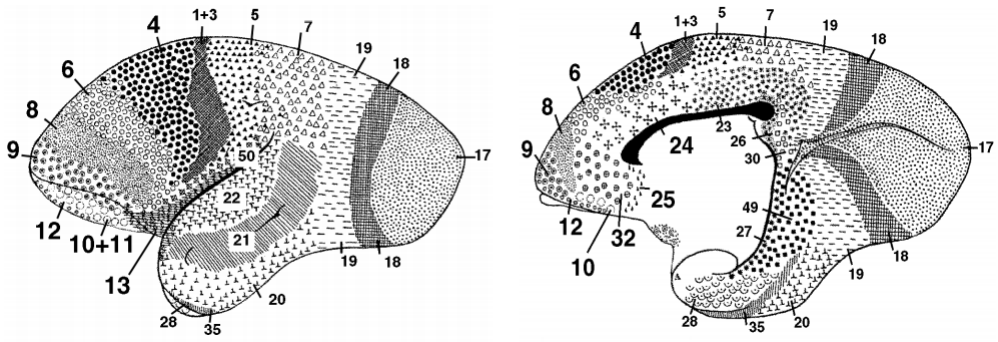


Figure 1-5 Brodmann's map of Hapale (now Callithrix) neocortex. In Brodmann's 1909 map, marmoset and macaque PFC contained identical cytoarchitectonic subregions. Included in the vmPFC are well defined BA24, 25 and 32.

1.1.2.3 Human ventromedial prefrontal cortex

Extensive work has focused on parcellating human cerebral cortex, including vmPFC. The first attempt at cerebral cartography of the human cortex was conducted by Alfred Campbell in the early 20th century (Campbell, 1905). By assessing changes in cellular architecture across the cortex, Campbell identified 17 fields including a limbic field along the cingulate gyrus consisting of three subzones – limbic 'A' (mid cingulate/dACC/pgACC), 'B' (sgACC) and 'C' (posterior cingulate) (**FIGURE 1-6A**). In comparison to Campbell's 17 fields, Brodmann recognised 44 cerebral divisions using more detailed cytoarchitectural techniques. Whilst not considered part of the PFC at the time, BA10, BA25, and BA32 were recognised as comprising the medial wall (**FIGURE 1-6B**). Brodmann's classification system is still the most widely used in humans, in part because of the (arbitrary) decision to adopt Brodmann's nomenclature in the influential Talairach-Tournoux neuroimaging atlas (Talairach and Tournoux, 1988).

In parallel to Brodmann's cell-based approach, Cecile and Oskar Vogt adopted a myeloarchitectonic approach to parcellate human cerebral cortex (Vogt, 2015; Vogt and Vogt, 1919) (**FIGURE 1-6C**). Over 200 areas were identified, many of which are subdivisions of Brodmann's fields. Their mapping of the cingulate gyrus was particularly extensive and detailed. In their work, the cortex of the anterior cingulate was divided into 22 subregions including three regions corresponding to BA25, nine regions corresponding to BA32 and ten regions spanning BA24 and parts of BA32. Because their work remained largely incomplete for the temporal and occipital cortex, their myeloarchitectural map has not been extensively used.

Following this work, von Economo and Koskinas published a revised map of the human cortex based upon their own cytoarchitectonic analysis (von Economo & Koskinas, 1925) (**FIGURE 1-6D**). A similarly gargantuan effort, von Economo and Koskinas identified 107 cortical areas as opposed to the 44 identified by Brodmann. In the medial wall, a region was identified similar in position and extent to BA25. However, BA32 was no longer considered homogeneous and instead consisted of four separate subfields. Their work is considered by many to be the definitive text on cortical mapping; however, it never received widespread usage (likely owing to the encyclopaedic nature of the final text – 810 pages with 112 microphotographs).

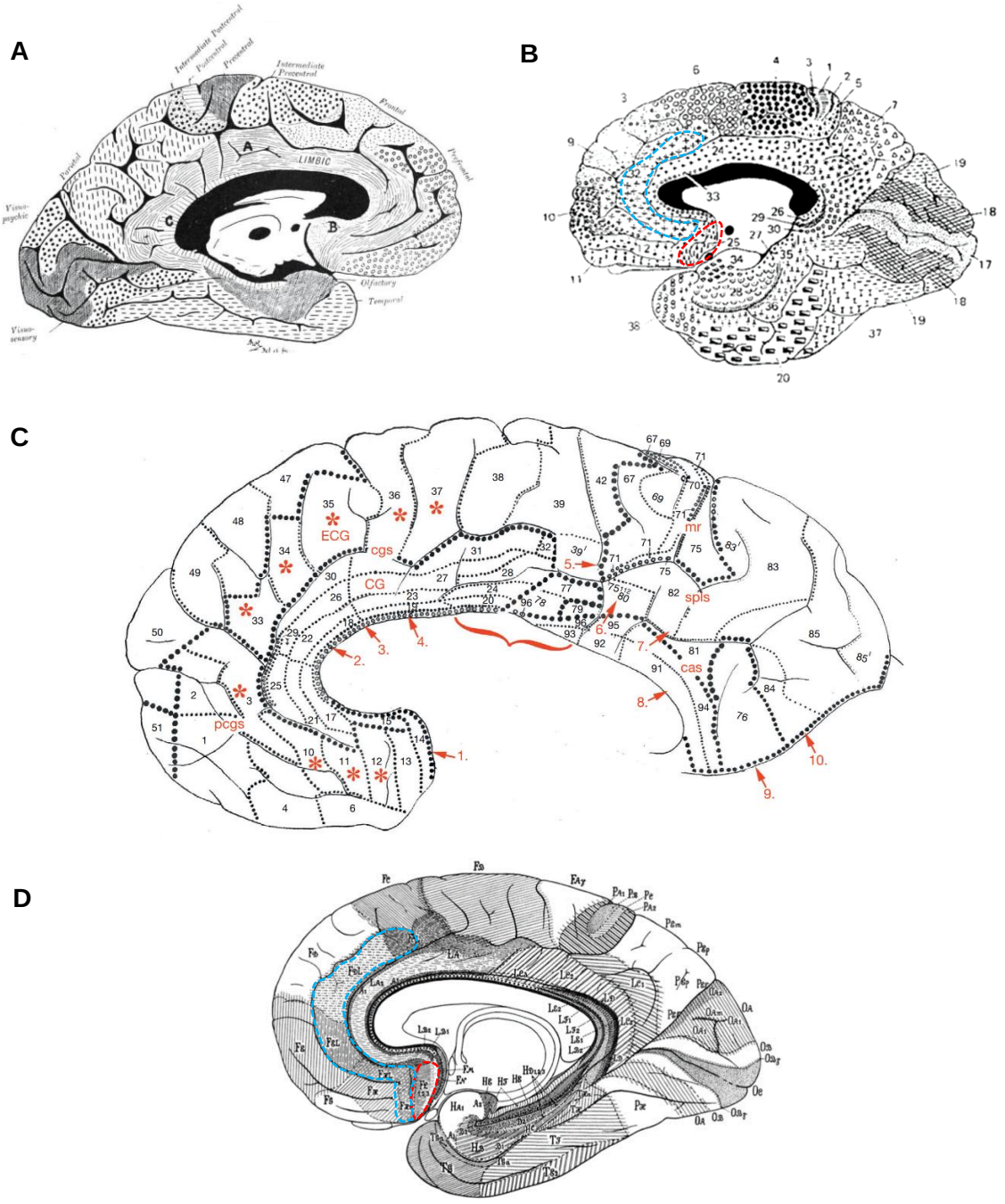


Figure 1-6 Maps of the medial wall of human cerebral cortex. **A** Alfred Campbells' 1905 map, showing limbic A (BA24/32), B (BA25) and C (posterior cingulate) subzones. **B** Brodmann's 1909 map. Highlighted in red, BA25, and in blue, BA32. **C** Vogt and Vogt's 1919 map, taken from Vogt, 2015, showing detailed characterisation of the cingulate gyrus. Note the two vertical and one horizontal divisions of BA25 (1.) and the nine divisions of BA32 (*). The dACC/BA24 is divided horizontally (2.) and delineated clearly from mid-cingulate (3.). **D** von Economo and Kosnikas' 1925 map. Highlighted in red is Ff, roughly equivalent to BA25. Highlighted in blue are FDL, F8L, FæL and FæF – four subregions which roughly correspond to BA32.

In the late 1990s/early 2000s, more precise characterisations of the subregions of human mPFC were developed. In 1994, whilst developing their map of macaque PFC, Petrides and Pandya developed a map of human PFC based on detailed cytoarchitectonic analysis (**FIGURE 1-7A**). At the time, Petrides and Pandya were trying to reconcile differences between human and monkey PFC literature, so findings from experimental research in the macaque could be more closely linked to structural and functional findings from human neuroimaging. In their human map, BA32 and BA24 comprise the majority of dACC and pgACC, but also have extensive portions in the sgACC. Human BA25 is restricted to caudal sgACC (note that in their macaque map, BA25 constitutes the majority of rostral and caudal sgACC). Öngür and colleagues built upon this work by using similar techniques Carmichael and Price had employed in the macaque – namely multi-modal cyto-, myelo- and chemoarchitectural classification systems – to fractionate human vmPFC (Öngür et al., 2003) (**FIGURE 1-7B**). BA32 was found to be heterogeneous: the 'human' BA32 (equivalent to the human region identified by Brodmann) was labelled as 32ac (ac, anterior cingulate) and located perigenually, whereas 'monkey' BA32 (equivalent to the monkey region identified by Brodmann) was labelled 32pl (pl, prelimbic) and located in sgACC. Notably, both maps in **FIGURE 1-7** omit parcellation of BA24.

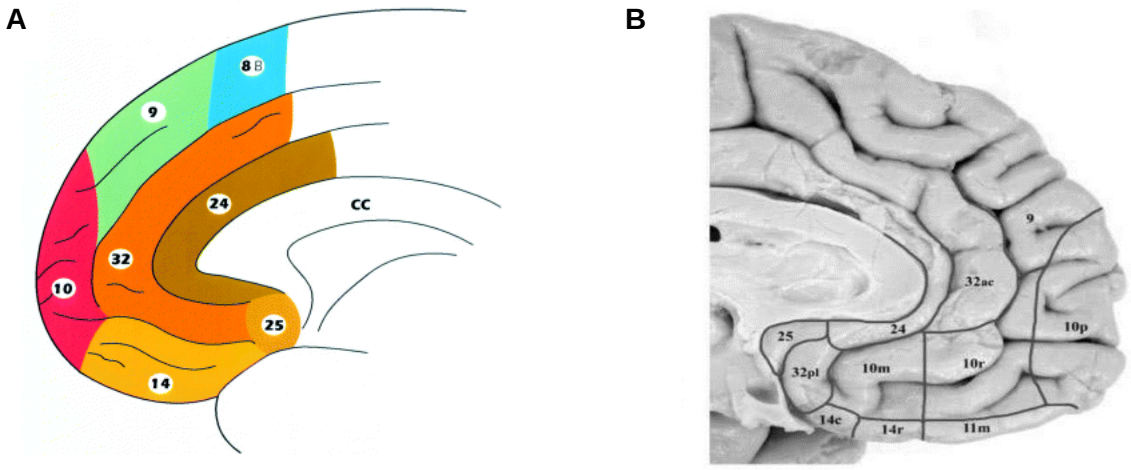


Figure 1-7 Maps of the human mPFC based on multi-modal classification approaches. **A** Petrides and Pandya, 1994. **B** Öngür et al., 2003. Both maps were developed in the context of comparing human mPFC with macaque mPFC using a combination of cyto-, myelo- and chemoarchitectural approaches.

Neuroanatomical techniques are constantly developing and improving, and consequently classification systems are continuously revisited and revised. Most recently, Palomero-Gallagher *et al.* have employed an anatomical and functional approach to precisely classify subregions within human sgACC (Palomero-Gallagher et al., 2015) (**FIGURE 1-8**). Using

macroscopic landmarks, cyto- and chemoarchitectural techniques together with functional connectivity profiles during tasks involving reward, punishment or fear processing, they propose four distinct fields: 25, s24, s32 and ventral 33. These regions have different functional connectivity profiles: s24 was associated with sadness; s32 with fear processing; 33 with painful stimuli; and 25 with regulation of autonomic and endocrine functions, together with any tasks involving reward perception.

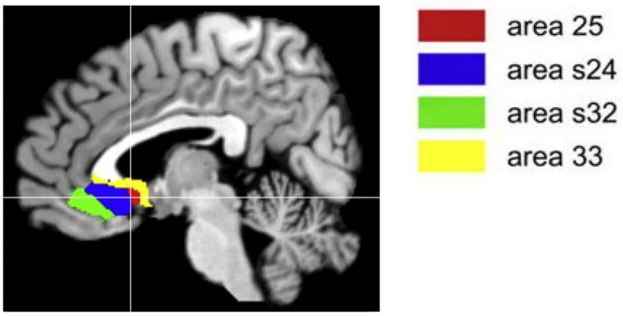


Figure 1-8 Functional organisation of human sgACC. Taken from Palomero-Gallagher *et al.* (2015). Sagittal section showing the locations of area 25, s24, s32 and 33 based on functional activation studies and anatomical features. The different sgACC subzones show distinct anatomical features, different task-dependent functional connectivity profiles and are associated with different functions.

1.1.2.4 Rodent-primate homology

When Vogt and Peters evaluated the cytoarchitecture of the rat cingulate cortex in 1981, they identified divisions corresponding to those identified by Brodmann in humans/NHPs – they therefore adopted Brodmann's nomenclature (Vogt and Peters, 1981). In 2014, Vogt and Paxinos revised this map in an attempt to clarify and reaffirm the homology between human and rodent (Vogt and Paxinos, 2014). Using cytoarchitectural techniques combined with differences in receptor architecture, intra-cingulate connectivity and ligand binding, they confirmed the presence of clear homologies between human and rodent anterior cingulate zones including BA24 (AC), 25 (IL) and 32 (PL). Their human-rodent mapping is shown in **FIGURE 1-9**. Based on their work, they divided human BA32 into four divisions. Two of these can be homologised to the rodent: perigenual (p)32 and subgenual (s)32 are found in primates, which are homologous to dorsal (d)32 and ventral (v)32 respectively in rodents (p32 and s32 are also roughly equivalent to 32ac and 32pl identified in (Öngür et al., 2003)). In both primates and rodents, p/d32 have a dysgranular layer IV, whereas s/v32 have large and dense neurons in layer V.

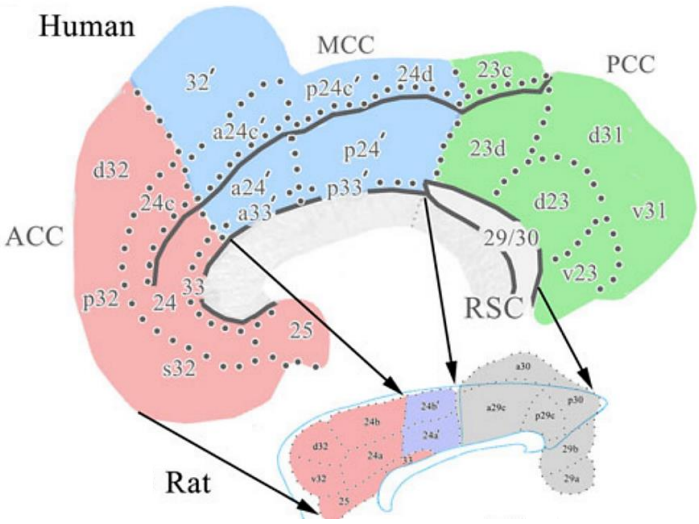


Figure 1-9 Vogt and Paxinos' 2014 map, highlighting homology between human and rodent mPFC. Anterior, mid and posterior divisions of the cingulate cortex are highlighted in red, blue and green respectively (note: rodents do not have a posterior cingulate region – instead, their retrosplenial cortex [RSC] is expanded and constitutes a much larger portion of the medial wall).

Most recent work by Heilbronner, Haber and colleagues has largely corroborated this anatomical homology through examination of cortico-striatal projections (Heilbronner et al., 2016). In their study, they defined the 'striatal emotional processing network' (EPN) as the projections to the accumbens shell, together with hippocampal- and amygdala-striatal projection zones. The striatal EPN is conserved across species, so by examining overlap between the striatal EPN and projections of vmPFC subregions, Heilbronner *et al*. could use these conserved striatal features to determine homology. Based on this analysis, the overlap between IL-BA25 and PL-BA32 projection zones and the EPN was very similar. Using these precision anatomical approaches, Heilbronner and colleagues have corroborated the suggestion that IL-25 and PL-BA32 are anatomically homologous. Whether anatomical homology necessitates functional analogy, however, remains unclear.

1.1.2.5 A note on BA14: ventromedial prefrontal cortex vs. medial orbitofrontal cortex

Discussion so far has focused on the anatomy medial wall of the prefrontal cortex – BA10, 14, 24, 25 and 32. As with BA25, BA14 is present on the medial wall but also extends onto the orbital surface of the PFC in NHPs and humans (Öngür et al., 2003; Petrides and Pandya, 1994) and is therefore variably considered part of the vmPFC or part of mOFC. Furthermore, in many human functional neuroimaging studies, changes detected in regions including BA14 are variably discussed as being part of vmPFC or mOFC. For the purposes of further discussion in this thesis, studies examining the contribution of BA14 will also be considered when discussing function of the vmPFC.

1.2 Emotion, Cognition and the Ventromedial Prefrontal Cortex

Parallel perspectives have emerged which emphasise the function of the vmPFC in (i) reward processing and value-based decision-making; (ii) the regulation of negative emotion; and (iii) social cognition (Hiser and Koenigs, 2018).

1.2.1 Ventromedial prefrontal cortex in reward processing and value-based decision-making: evidence from animals

1.2.1.1 Evidence from rodents: vmPFC contributions to appetitive Pavlovian and instrumental processes

1.2.1.1.1 Rodent vmPFC in appetitive Pavlovian conditioning

Very few studies have investigated the contributions of rodent IL/PL to the acquisition or expression of Pavlovian appetitive responses. In one recent study, the effects of IL inactivations were assessed following acquisition of an appetitive Pavlovian response (food port entry) to an auditory conditioned stimulus (CS) (Mendoza et al., 2015). Whilst IL inactivations had no effect on responses during the CS, there were many more entries during the baseline (pre-CS) period, and even during sessions where the CS was never presented. This may be interpreted as an impulsive 'checking' behaviour, akin to the impulsive behaviour observed on the 5-choice serial reaction time task following IL lesions (Chudasama et al., 2003) – *i.e.* not directly related to appetitive learning *per-se*. To my knowledge, there have been no studies examining the role of PL in appetitive Pavlovian conditioning.

Similarly, few studies have assessed the contributions of rodent vmPFC to the extinction of appetitive Pavlovian memories. Rhodes and Killcross used a Pavlovian conditioned approach assay to demonstrate that whilst IL lesions did not affect the extinction of appetitive conditioned responses, there was enhanced spontaneous recovery and reinstatement on subsequent days (Rhodes and Killcross, 2004). It has since been suggested that IL acts as a source of projections to the nucleus accumbens shell, mediating the extinction of appetitive memories (Peters et al., 2009). In the study by Mendoza and colleagues mentioned above, pharmacological inactivation of IL was also shown to result in more rapid extinction of appetitive Pavlovian memories with no effect on extinction recall (whereas PL inactivation had no effect) (Mendoza et al., 2015). This implicates IL in maintaining appetitive Pavlovian responding when appetitive USs are omitted – although it is at odds with the majority of rodent fear extinction literature which suggests that IL inactivation *impairs* extinction (see **1.2.3.1.1**). It is also at odds with a more recent study, showing that pharmacological and optical stimulation of IL during CS presentations in extinction recall reduce the reinstatement of CS-elicited port-entries (Villaruel et al., 2017). These data would suggest that IL inhibits appetitive Pavlovian responding following extinction. Given the variation in findings between

different studies (e.g. Mendoza *et al*. *vs.* Villaruel *et al.*), it seems that the precise role of IL remains to be determined. Interpretation is complicated by the different approaches used in different laboratories, which manipulate IL either before extinction, before extinction recall or during extinction recall.

1.2.1.1.2 Rodent vmPFC in appetitive instrumental conditioning

A more extensive body of work has explored the role of IL and PL in appetitive instrumental behaviours. Emergent from this work is the hypothesis that IL and PL have differential roles in goal directed *vs.* habitual responding for reward. Lesions of IL prevent the development of a S-R habit; despite overtraining, rodents are still sensitive to reward devaluation (a hallmark of goal-directed behaviour) (Killcross and Coutureau, 2003). Lesions of PL have the opposite effect, reducing sensitivity to reward devaluation. This has led to the suggestion that PL lesioned animals are 'creatures of habit' (Balleine and Dickinson, 1998; Killcross and Coutureau, 2003). Interestingly, Coutureau and Killcross have shown that inactivations of IL can reinstate goal-directed responding following over-training (Coutureau and Killcross, 2003), suggesting that goal-directed behaviours are actively inhibited when responding is habitual.

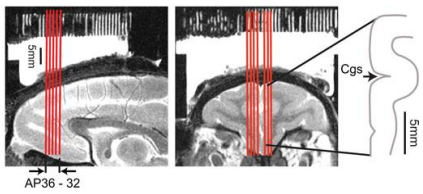
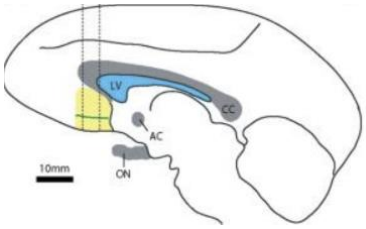
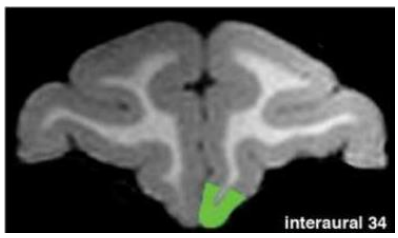
Rodent vmPFC has also been implicated in the extinction of appetitive instrumental memories, however the data are once again discrepant. Studies assessing the role of IL in response suppression following extinction have shown that manipulations reducing IL activity promotes reinstatement of appetitive instrumental responding (Peters et al., 2008; Warren et al., 2016). Consistent with these findings, enhancing IL activity using pharmacological (Chen et al., 2016; Peters et al., 2008) or chemogenetic (Augur et al., 2016) techniques inhibits the reinstatement of drug-seeking behaviour. LaLumiere and colleagues additionally showed that IL inactivation immediately *after* extinction training impairs extinction recall (LaLumiere et al., 2010) suggesting a role for IL in the consolidation of extinction memories. These data would suggest that response suppression after extinction is maintained by activity within IL.

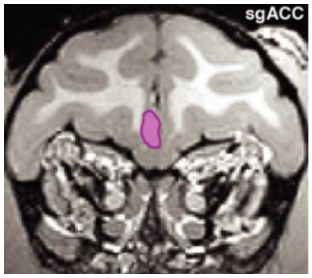
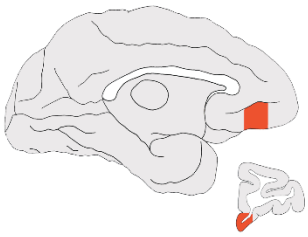
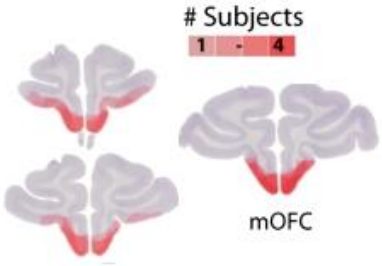
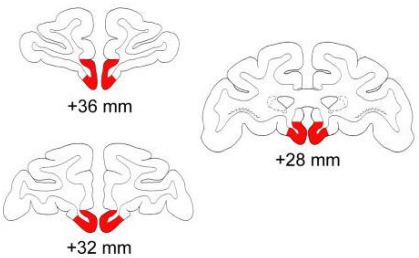
However, several inactivation studies have found that inactivating IL – rather than augmenting responding – reduces (Bossert et al., 2011; Eddy et al., 2016; Rogers et al., 2008) or has no impact (Willcocks and McNally, 2013) on the return of operant behaviour during extinction recall. One study which selectively ablated neurons in IL activated by a heroin-associated context found that this manipulation *reduced* (rather than enhanced) the context-induced renewal of heroin-seeking behaviour (Bossert et al., 2011). The reasons for these discrepancies are unclear. One possible reason again relates to the timing of IL manipulations: for example, inactivating IL immediately after extinction may disrupt *consolidation* of an appetitive extinction memory, whereas inactivating IL on the extinction recall day does not disrupt the *expression* of an extinction memory in the same way. Fewer

studies have assessed the role of PL in appetitive instrumental extinction, although Sparta *et al.* have used optogenetic techniques to stimulate fast-spiking inhibitory interneurons within PL (thereby inhibiting PL output) and found that extinction is accelerated following PL inhibition (Sparta et al., 2014).

1.2.1.2 Evidence from non-human primates: vmPFC contributions to subjective valuation, reward-based decision-making and reward anticipation

The combined use of electrophysiological, lesion and neuroimaging approaches has implicated subregions of NHP o/vmPFC in reward-related constructs. For a summary of studies implicating NHP vmPFC (and subregions of mOFC) in reward-related processing, see **TABLE 1-1**.

Reference	Image	Description
	Electrophysiological studies	
(Amemori and Graybiel, 2012)	 BA32	Electrophysiology and microstimulation, macaques: along its dorsoventral extent, pgACC/32 contains intermixed groups of neurons which represent both motivationally positive and negative subjective value; in one subregion along this axis, there is a particularly concentrated focus of negative-encoding neurons which negatively bias reward-based decision making through over-estimation of costs.
(Monosov and Hikosaka, 2012)	 BA14, 25	Electrophysiology, macaques: recorded 'dorsal'-vmPFC neurons (roughly BA25) and 'ventral'-vmPFC neurons (roughly BA14) and found dorsal neurons were persistently more active in aversive blocks of a Pavlovian task (signalling CS and US), whereas ventral neurons were persistently more active in appetitive blocks (signalling CS and US)
(Strait et al., 2014)	 BA14	Electrophysiology, macaques: recorded vmPFC neurons whilst macaques performed a gambling task. vmPFC neurons show (i) tuning for reward probability and reward size (i.e. expected value signal); (ii) inversely correlated tuning curves for the two decision options suggesting they are under mutual inhibition; and (iii) rapid

		convergence following choice to signal the value of a chosen offer.
(Azab and Hayden, 2018)	 BA25	Electrophysiology, macaques: neurons in sgACC encode multiple aspects of reward processing, responding especially to losses and anticipation of primary rewards. Many of these encoding properties were also seen in the dACC.
(San-Galli et al., 2018)	 BA14r	Electrophysiology, macaques: recorded vmPFC neurons in monkeys squeezing a grip for fluid rewards. vmPFC neuron activity was closely related to slow changes in motivational state (fatigue, satiety) and reliably predicted monkey's willingness to perform the task.
	<u>Lesion studies</u>	
(Noonan et al., 2010)	 BA14	Aspiration lesion, macaque: mOFC-lesioned animals are impaired during reward-guided decision making. Specifically, animals are more susceptible to errors when deciding between two options close together in value (or when deciding between two options disparate in value, with a third distractor option). Note that mOFC includes the portion of BA14 extending onto the medial wall.
(Rudebeck and Murray, 2011)	 BA14	Excitotoxic lesion, macaques: lesions of BA14 prevent ability of monkeys to stop responding to a previously rewarded object during extinction.

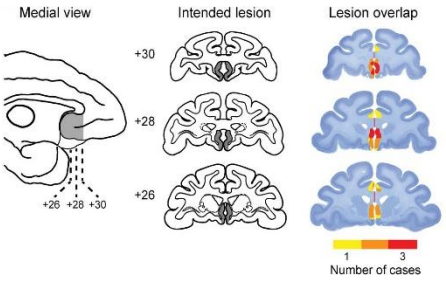
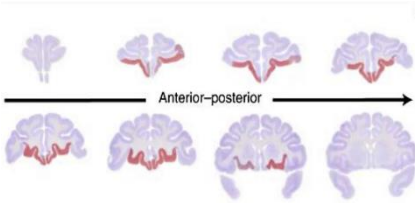
(Rudebeck et al., 2014)	 BA25	Ablative lesion, macaques: ablation of BA25 in macaques impairs monkeys' ability to sustain autonomic arousal (as measured by pupil diameter) in a trace interval between an appetitive CS and US.
(Papageorgiou et al., 2017)	 BA11m, 13, 14	Ablative lesion and fMRI, macaques: vmPFC lesions do not impair the ability to learn simple stimulus-reward associations but do alter the subjective valuation of reward in more complex situations. Activity in vmPFC reflects the subjective value difference between a choice taken and a choice rejected.

Table 1-1 Studies implicating primate vmPFC (and subregions of mOFC) in reward-related processing. Electrophysiological and lesion studies presented separately. Electrophysiological studies evidence functional heterogeneity even within the same BAs – neurons in BA14 appear broadly tuned for different aspects of reward processing, whereas neurons in BA25 and BA32 are more intermixed with both aversive and appetitive functions. Lesion studies illustrate that lesions targeting BA14 and BA25 appear to impair reward processing. BA14 lesions have been shown to affect instrumental reward-based decision making, and BA25 lesions impair the maintenance of autonomic arousal during a trace interval. There is a dearth of studies investigating the casual contributions of subregions of NHP vmPFC to reward processing, limiting the scope of any conclusion that can be drawn.

Electrophysiological studies have, in all cases, revealed specialisation and heterogeneity in the reward-encoding properties of neuronal populations. Whether there is any general organising principle is not clear – although the general encoding properties of neurons seems to vary between different subregions, there is still variation within single BAs. For example, neurons within BA32 heterogeneously encode positive and negative offer values and choices (Amemori and Graybiel, 2012), whereas activity of neurons in BA25 is related to the anticipation of positive and negative outcomes (Azab and Hayden, 2018; Monosov and Hikosaka, 2012). Still more ventrally, neurons in BA14 seem to encode subjective value

signals, fire during the anticipation of reward and show slower changes reflecting changes in motivational state (Monosov and Hikosaka, 2012; San-Galli et al., 2018; Strait et al., 2014).

Results of lesion studies similarly implicate the vmPFC in reward processing, particularly BA14. BA14 has a role in representing subjective value (Papageorgiou et al., 2017) and in reward choice (Noonan et al., 2010). Lesions of BA14 in the macaque impair the extinction of instrumental appetitive memories (Rudebeck and Murray, 2011) in a similar fashion to lesions of IL in rodents (Rhodes and Killcross, 2004). Only one study has assessed the impact of BA25 lesions in macaques in the context of reward arousal. Rudebeck and colleagues demonstrated that macaques with BA25 lesions were unable to sustain autonomic arousal (pupil diameter) during a trace interval introduced between a CS and rewarding US (Rudebeck and Murray, 2011). Note that these were ablations, and therefore the effects could be due to damage to underlying fibres of passage.

1.2.2 Ventromedial prefrontal cortex in reward processing and value-based decision-making: evidence from humans

Ever since Harlow's famous descriptions of the impairments in Phineas Gage's behaviour associated with bilateral damage to o/vmPFC following an accident with a tamping iron (Harlow, 1868), ventral regions of the PFC have been appreciated as playing a critical role in affective behaviour. After the lesion, Gage showed profound deficits in social and emotional behaviour – Harlow described Gage as follows:

> *"He is fitful, irreverent … [and] impatient of restraint or advice when it conflicts with his desires … Gage was no longer Gage."* (Harlow, 1868)

Although highly informative, conclusions drawn from this case must be tempered with realism: first, it is a single case report, and second, damage involved several subregions within the PFC (including BA8-10, BA24 and BA32) making it impossible to attribute the impairments with a particular subregion (Damasio et al., 1994). Subsequently, patients with damage more localized to the vmPFC have been characterised as having impairments in value-based and social decision-making with preserved 'general intelligence,' consistent with the hypothesis that ventromedial regions have a role in these functions (Barrash et al., 2000; Eslinger and Damasio, 1985).

At the time of Harlow's account, the alterations in Gage's behaviour were not specifically linked to impairments in reward processing. More precise characterization of the vmPFC 'syndrome' in reward-related behaviour was achieved following the development of gambling tasks such as the Iowa Gambling Task (IGT) and Cambridge Gambling Task (CGT). In these tasks, participants learn about reward and punishment contingencies under conditions of ambiguity (Bechara et al., 1994, 1999). vmPFC-lesioned patients typically show increased

betting under conditions of higher risk (with unimpaired declarative performance in judging probabilities), consistent with a role of intact vmPFC in biasing individuals towards safer, conservative options during uncertainty.

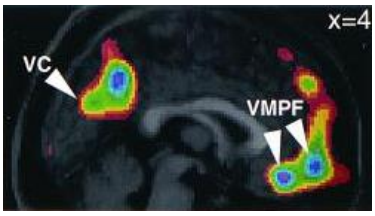
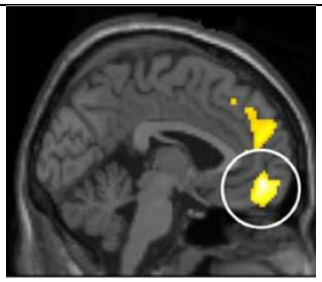
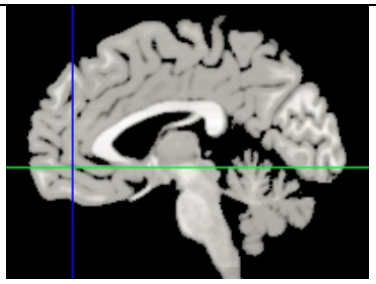
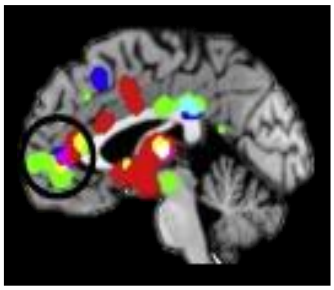
Following on from these studies, two perspectives have emerged: (i) the vmPFC mediates the affective ('gut-feeling') contribution to value-based decision-making (based largely on lesion studies); and (ii) the vmPFC represents and updates the reward values of incentive stimuli and primary outcomes (based largely on functional neuroimaging studies). These perspectives are not mutually exclusive; rather, they differentially emphasise a role for vmPFC in 'hot' (emotion-laden) *vs.* 'cold' (emotion-independent) cognitive aspects of decision-making.

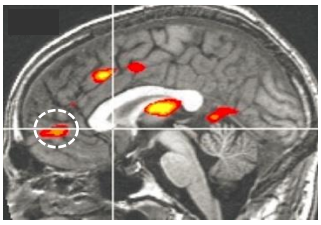
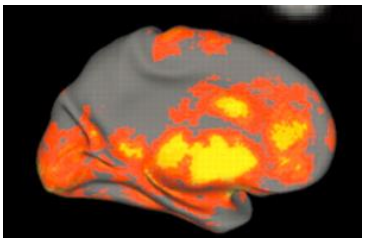
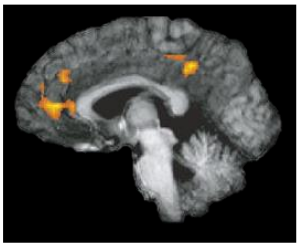
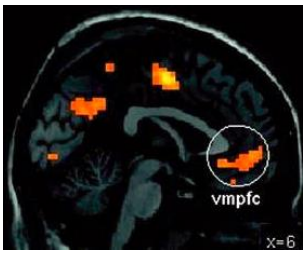
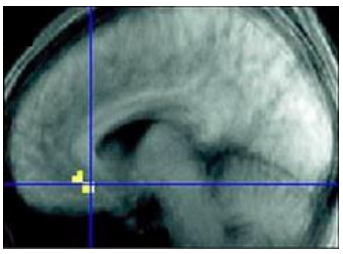
1.2.2.1 vmPFC and 'hot' cognition: somatic maker hypothesis

A role for vmPFC in mediating the affective 'gut-feeling' component of decision-making was developed by Antonio Damasio in the 1990s, termed the somatic marker hypothesis (Damasio, 1996). Damasio's aim was to understand the decision-making deficits observed in Phineas Gage and other patients with vmPFC damage. He emphasised that emotions are typified by physiological (visceral) and behavioural (musculoskeletal) changes, together with subjective changes in feelings. The changes in the "*musculoskeletal, visceral and internal milieu components of the soma*" comprised a 'somatic state.' The vmPFC served as a privileged locus by having access to the body's current somatic state (through connections with the insula), together with sensory and contextual information about an individual's current situation (connections with sensory cortex). The vmPFC links specific internal somatic states to external stimuli/situations and can reactivate somatic states when these stimuli/situations are encountered in the future. Reactivation of these states occurs rapidly and subconsciously, and feedback from the periphery can then bias the decision-making functions of the vmPFC based on the history of reward in those specific contexts. Under conditions of uncertainty where multiple variables influence reward probability in complex, non-linear ways (such as IGT/CGT), 'cold' cognitive cost-benefit decision-making is too slow to compute the likelihood of an outcome. In such situations, 'gut-feelings' (reactivation of appropriate somatic markers by the vmPFC) are critically important to rapidly bias decision-making. Whilst attractive in its ability to explain behavioural performance and autonomic responsivity of vmPFC-lesioned patients on IGT/CGT (Bechara et al., 1994, 1996), the somatic marker hypothesis is not without criticism. For instance, whether the contingencies during the IGT/CGT are truly 'ambiguous' has been questioned, with the suggestion that these tasks are highly cognitively-penetrable and do not reflect a contribution of subconscious bias ('gut feeling') to decision-making (Dunn et al., 2006).

1.2.2.2 vmPFC and 'cold' cognition: reward valuation

In parallel, a large body of work has related subjective value judgements, the anticipation of reward and various aspects of reward-based decision-making to functional magnetic resonance imaging (fMRI)-measured blood oxygen level dependent (BOLD) signals throughout the brain. Across multiple different tasks and reward types these studies have yielded relatively homogeneous results, with almost all of them identifying activity within the vmPFC correlated with subjective reward value (together with the striatum, amygdala and insula) (Levy and Glimcher, 2012). In several cases, BOLD responses across different reward types have been measured within the same study, facilitating the use of conjunction analysis to identify brain regions which encode reward value across different modalities. These consistently show vmPFC activation confined to a very similar cluster (e.g.) (Chib et al., 2009; FitzGerald et al., 2009; Kim et al., 2011; Lin et al., 2012). Activity within vmPFC also spans different constructs related to reward processing – seen in reward anticipation; during rewarding outcomes; and in reward-based decision-making scenarios. See **TABLE 1-2** for studies showing human vmPFC activations spanning different aspects of reward processing, including subjective valuation, reward anticipation and reward-based decision making.

Reference	Image	Description
	Activation to rewarding outcomes	
(Blood and Zatorre, 2001)	 BA10, 11, 14	vmPFC activation associated with intensely pleasurable responses to music.
(Rolls et al., 2003a)	 BA10, 11	vmPFC activation correlating with the subjective rating of pleasantness of three different pleasant odours.
(de Araujo et al., 2003)	 BA10, 11	vmPFC activation ('caudal OFC') when thirsty humans drink water – the activation was not present when the same participants drank water once sated.
(Chib et al., 2009)	 BA10, 32	[Location of peak activation] Region of vmPFC correlated with subjective value across different categories of goods, including food, non-food consumables and monetary gambles.
(Sescousse et al., 2013a)	 BA10, 24, 32	[meta-analysis] vmPFC region showing overlapping activation to monetary, food and erotic rewards.

Activation in anticipation of rewards		
(Knutson et al., 2005)	 BA10	vmPFC activity correlates with probability estimates of future reward during the anticipatory phase of the MID task.
(Tom et al., 2007)	 BA10, 11, 24, 25, 32	Region of vmPFC showing increasing activity as potential gains increase during a gambling task (same region shows decreasing activity as potential losses increase).
(Kable and Glimcher, 2007)	 BA10, 24, 32	Region of vmPFC tracking the subjective value of delayed monetary rewards.
(Gläscher et al., 2009)	 BA10, 25, 32	Activity in vmPFC tracks expected future reward during action-based and stimulus-based reward decision making task.
(Kim et al., 2011)	 BA10, 25	vmPFC activation overlapping between conditions of expecting juice reward and expecting monetary reward.

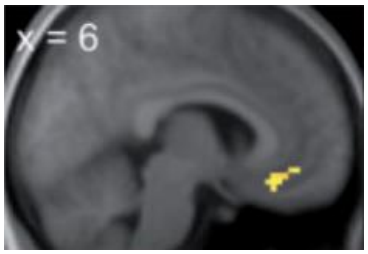
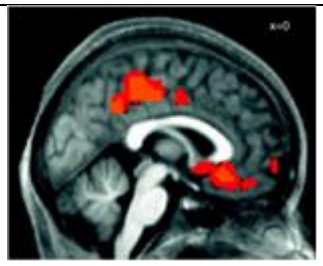
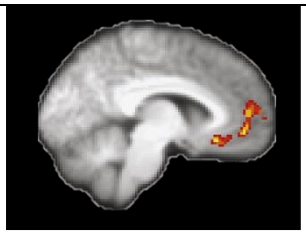
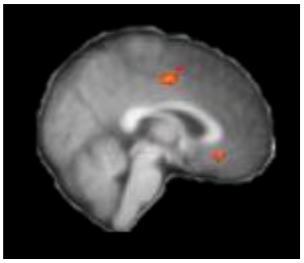
(Lin et al., 2012)	 BA10	vmPFC activation overlapping between conditions of expecting social reward (pictures of smiling people) and expecting juice reward.
Activation at choice during reward-based decision making		
(FitzGerald et al., 2009)	 BA10, 24, 25, 32	vmPFC activation reflects the difference in subjective value when comparing incommensurable outcomes.
(Boorman et al., 2009)	 BA10, 32	vmPFC encodes relative chosen value between two options (chosen – unchosen expected value).
(Boorman et al., 2013)	 BA10	Activity at choice in vmPFC is tied to the value of the current choice.

Table 1-2 Human vmPFC activations related to reward processing. Activations have been grouped broadly according to (i) activations during rewarding outcomes, (ii) activation during the anticipatory period before reward receipt and (iii) activations at choice phase during decision making. It is apparent that a highly similar region of rostral vmPFC is activated across these different aspects of reward processing – typically corresponding to BA10, occasionally extending posteriorly into BA25 and dorsally into BA24/32.

1.2.3 Ventromedial prefrontal cortex in the regulation of negative emotion: evidence from animals

The second critical function in which vmPFC plays a major role is the regulation of negative emotion, and much of our understanding of this function comes from work in rodents and non-human primates.

1.2.3.1 Evidence from rodents: fear conditioning and extinction; fear generalisation; and the controllability of stress

1.2.3.1.1 Fear conditioning and extinction

Using fear conditioning and extinction paradigms, rodent studies have causally implicated the vmPFC in regulating negative emotion. In these paradigms, rodents acquire a Pavlovian CS/US association (e.g. tone-shock; 'acquisition'), extinguish it through non-reinforced CS presentations (tone-no shock; 'extinction') and are then tested for successful extinction the following day through further presentations of the non-reinforced CS (tone-no shock; 'extinction recall'). The acquisition of the CS/US association involves increased expression of the conditioned response (CR) – freezing in the case of rodents. Extinction involves reduction in the CR as the animal learns that the CS no longer predicts the US. Early theorists posited that fear extinction processes resulted in the weakening and eventual removal of the CS/US association that was learnt during acquisition (Rescorla and Wagner, 1972). However, phenomena such as spontaneous recovery of fear conditioning and accelerated re-acquisition provide evidence that the CS/US association is not simply 'unpaired' following extinction. Instead, it appears that learning of a new CS/noUS inhibitory association takes place (Bouton et al., 2006; Milad and Quirk, 2002).

Early experiments assessed the effects of broad lesions to rodent mPFC/vmPFC (including PL, IL, MO and AC) on the extinction of fear memories and found these lesions severely impaired extinction without an effect on acquisition (Morgan et al., 1993). Studies more restricted to vmPFC (IL and PL) then followed, highlighting a role for vmPFC in the successful recall of extinction (Quirk et al., 2000). In the same study, lesions of vmPFC which spared most of IL did not have an effect, suggesting that IL is the critical vmPFC sector necessary for recalling fear memories.

Following on from lesion studies, electrophysiological, microstimulation and pharmacological inactivation studies have probed the specific contributions of IL *vs.* PL in fear regulation. In seminal work, Milad and Quirk recorded from IL neurons during acquisition, extinction and extinction recall phases and found that IL neurons fire only when recalling a CS/noUS association on extinction recall days (Milad and Quirk, 2002). The degree of firing correlated with successful recall of this association: the more IL neurons fired, the less rodents froze. The same study also demonstrated at least some degree of causality between IL neuron

activity and successful extinction, as brief 300ms microstimulation of IL during extinction (purportedly resembling recall-induced IL responses) resulted in lower freezing on both the day of extinction and during extinction recall. Work in 2004 extended upon this finding by showing that 300ms microstimulation of IL reduces conditioned freezing if given 0.1s after CS onset (the normal latency of CS-evoked responses), but has no effect if given 1s before or 1s after CS onset (Milad et al., 2004). The temporally-specific nature of the effects of IL stimulation suggests that IL inputs serve to gate the output of downstream structures associated with fear expression such as the amygdala (see below). Pharmacological inactivation studies have extended knowledge gleaned from lesion and microstimulation work by causally implicating IL in extinction and extinction recall (Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011) placing IL as a key player in the inhibitory mechanisms suppressing amygdala responses to extinguished CSs during CS-noUS learning and retention.

The evidence for a causal role of IL in successful extinction and its recall has led to the investigation of plastic changes within IL and their relationship to extinction memory consolidation. These studies have shown that infusion of the amnestic agent anisomycin into IL after extinction blocks subsequent extinction recall (Santini et al., 2004). Probing at the molecular mechanisms further, blocking molecular components of classic long-term potentiation (LTP) – including NMDA receptors and mitogen-activated protein kinase (MAPK) – in IL immediately after extinction training causes a failure of extinction recall 24 hours later, suggesting an impairment in consolidation (Burgos-Robles et al., 2007; Hugues et al., 2004). Consolidation of extinction has also been shown to be contingent upon ribonucleic acid (RNA) synthesis within IL, supporting a role for plasticity in this region in fear learning (Mueller et al., 2008). In sum, these data suggest that disruptions to the apparatus of cellular plasticity and learning in IL can result in failure of consolidation of CS-noUS memories in the interval between extinction and extinction recall.

Distinct from IL, electrophysiological recordings from PL do not indicate neurons sensitive to extinction recall, nor does PL microstimulation influence the memory of extinction (Milad and Quirk, 2002). Nevertheless, PL microstimulation has been shown to increase conditioned fear expression and impair extinction without impairing retention (Vidal-Gonzalez et al., 2006). Furthermore, activity within PL neurons is directly correlated with expression of the CR (freezing) (Burgos-Robles et al., 2009). However, inactivation of PL prior to acquisition does not impair the learning of fear associations – whilst animals freeze less during acquisition, presenting the CS on the following day (a test of fear learning) shows that they freeze at normal levels (Corcoran and Quirk, 2007). The same manipulation prior to extinction depresses fear responses on the same day without influencing subsequent recall

(Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). These multiple streams of evidence suggest that, distinct from IL, PL plays a role in the *expression* of learned fear, but not fear learning per-se (either during acquisition or extinction).

These data have led to the development of models of fear regulation which posit that PL and IL have opposing roles on amygdala output to facilitate flexibility when responding to danger-associated cues (**FIGURE 1-10**)**.** Quirk and colleagues have demonstrated that IL gates transmission of action potentials from BLA to CeN, such that prestimulation of IL reduces the responsiveness of CeN neurons to afferent action potentials incoming from BLA (Quirk et al., 2003). The functional relationship between IL and amygdala output has been consolidated with anatomical data: anterograde tracers injected into IL highlight its extensive connectivity with amygdala subnuclei, preferentially targeting inhibitory interneurons within CeN together with (inhibitory) neurons in the intercalated cell masses (Mcdonald et al., 1996; Sotres-Bayon and Quirk, 2010; Strobel et al., 2015). By contrast, PL projects directly to the BLA, presumably to influence expression of the CR (Sotres-Bayon and Quirk, 2010). The differential anatomical connectivity of PL and IL support the hypothesis that these regions have different roles in the regulation of negative emotion at the level of the amygdala (Vertes, 2004).

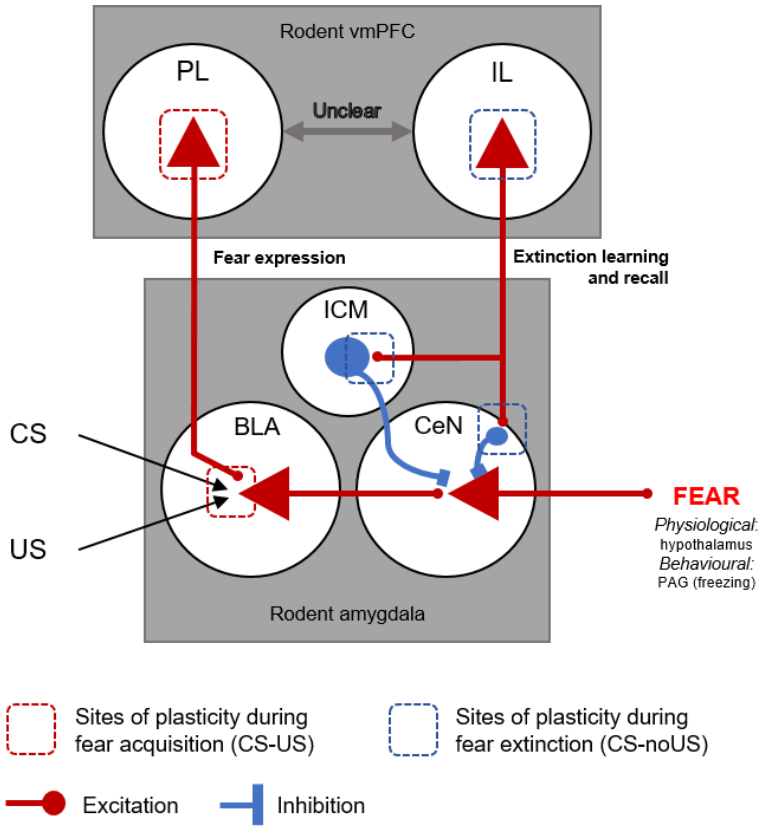


Figure 1-10 A model of PL and IL contributions to fear regulation by gating information flow in the amygdala. A wealth of anatomical and functional data (see text) supports the hypothesis that

subregions of rodent vmPFC – PL and IL – differentially regulate information flow within the amygdala. PL projects to BLA neurons to influence fear expression through downstream connections to the CeN, hypothalamus and striatum. The IL preferentially targets inhibitory interneurons, both within CeN and in the intercalated cell masses (ICM), to dampen output from the CeN. The CeN projects to the periaqueductal gray (PAG) to trigger behavioural aspects of the classic freezing response, and to the hypothalamus to trigger autonomic (including cardiovascular) and endocrine changes. During fear acquisition, neuronal plasticity is thought to occur at synapses from auditory (CS) and somatosensory (US) afferents (originating from both primary sensory cortex and sensory thalamus) into the BLA, such that over the course of learning, the strength of the CS-BLA synapse is strengthened. This means that CS presentation alone can drive a freezing response. There may also be plasticity within PL and at PL-BLA synapses, although evidence for this is lacking. During fear extinction (CS-noUS learning), plasticity has been shown to occur within IL itself and at IL-ICM synapses, supporting the suggestion of a functional relationship between this prefrontal subregion and inhibitory components of the amygdala during the acquisition/expression of an extinction memory.

1.2.3.1.2 Fear generalisation

A related but distinct line of work implicating rodent PL/IL in the regulation of negative emotion concerns the role of these regions in fear generalisation, although this field is still relatively nascent. In fear generalisation, animals express a learned fear response to a stimulus that is perceptually similar to a CS (Hiser and Koenigs, 2018). An initial study in 2012 showed the global abrogation of synaptic transmission in rodent mPFC (including PL, IL and AC) causes generalisation of fear memories (Xu et al., 2012). Subsequent work has shown that connections from the nucleus reuniens of the thalamus to the hippocampus, and from hippocampus back to vmPFC, seem to be necessary to prevent generalisation of fear in the presence of ambiguous stimuli (Xu and Südhof, 2013). It is important to highlight that these initial studies failed to differentiate between the functionally heterogeneous IL and PL. No rodent studies to date have addressed the potentially separable roles of IL and PL in fear generalisation.

1.2.3.1.3 Controllability of stress

Some aspects of stress 'controllability' – a composite construct including the *perception* of control, the *identification* of controllable situations and the exertion of *effortful* control (Kerr et al., 2012) – are uniquely human (Abramson et al., 1978), but rodent studies have provided insights into the role of IL/PL subregions in the behavioural correlates of a lack of control associated with learned helplessness. In learned helplessness models, animals learn that their attempts to escape a shock are futile. When animals are subsequently challenged with avoidable shock – typically in a shuttle-box apparatus – those that have been preconditioned with unavoidable shocks fail to acquire the instrumental avoidance response (Seligman,

1974). Maier and colleagues have shown that serotonergic output from the dorsal raphe nucleus (DRN) is necessary for mediating the deleterious behavioural consequences of learned helplessness on post-shock escape responses (Maier et al., 1993, 1995).

Anatomical studies indicate that input to the DRN is almost exclusively from IL/PL (Gabbott et al., 2003, 2005; Jankowski and Sesack, 2004). Pharmacological inactivation of IL/PL blocks the 'behavioural immunisation' effects of prior experience of control on the behavioural responses during instrumental avoidance (Amat et al., 2005, 2006), suggesting a critical role of rodent vmPFC in signalling the controllability of stress (Robbins, 2005) (note: in the Amat *et al.* studies, cannula placement in 2005 study is predominantly IL; cannula placement in 2006 study in IL/PL border zone). Congruently, activation of vmPFC (both IL and PL) using microinfusions of picrotoxin can mimic the stress resistance conferred by prior control experience (Christianson et al., 2009). Beyond the DRN, the beneficial inhibitory effects of vmPFC activity during stressful situations has been shown to involve numerous brain regions including the amygdala, suggesting that top-down feedback by the vmPFC has wide-ranging effects on limbic and paralimbic structures to mediate resilient behaviour (Maier et al., 2006).

1.2.3.2 Evidence from non-human primates: opposing roles of primate areas 25 and 32 and their putative rodent homologues in the regulation of negative emotion

[Work described in this paragraph was published in *Proceedings of the National Academy of Sciences, USA* in 2017 with the present author as a co-author: (Wallis et al., 2017)] The importance of NHP studies as a translational step to humans is nowhere more evident than when addressing the issue of vmPFC function in negative emotion. As discussed in **1.1.2.4**, it is generally accepted that the anatomical homologues of rodent IL and PL are primate vmPFC subregions area 25 (part of the sgACC; referred to hereafter as sgACC/25) and 32 (part of the pgACC; referred to hereafter as pgACC/32). These homologies have been established owing to a wealth of anatomical data demonstrating similarities in cytoarchitecture together with similar connectivity of IL-sgACC/25 and PL-pgACC/32 to cortical, subcortical and striatal targets (Heilbronner et al., 2016; Joyce and Barbas, 2018; Vertes, 2004; Vogt and Paxinos, 2014).

Pharmacological interventional studies have recently been carried out in marmosets, facilitating causal manipulations of primate sgACC/25 and pgACC/32. This research is invaluable as its utility applies for both forward-translation of preclinical results to humans (marmoset vmPFC subregions are highly homologous to those of humans) and back-translation for comparison with rodent studies (comparing results from manipulating IL-sgACC/25 and PL-pgACC/32). One such study co-authored by the present author has illustrated that sgACC/25 inactivation reduces the behavioural and cardiovascular correlates of negative emotion during fear conditioning and fear extinction whereas pgACC/32

inactivation increases these correlates via generalisation (Wallis et al., 2017). At a coarse level, this suggests that NHP sgACC/25 may normally act to promote negative affect, whereas pgACC/32 reduces negative affect. These data conflict with rodent fear-conditioning studies outlined above, where inactivation of IL (anatomical homologue of sgACC/25) increases negative affect whereas inactivation of PL (anatomical homologue of pgACC/32) reduces negative affect. Anatomical homology, therefore, does not appear to translate to functional similarity between these prefrontal regions when assessing fear-related behaviours.

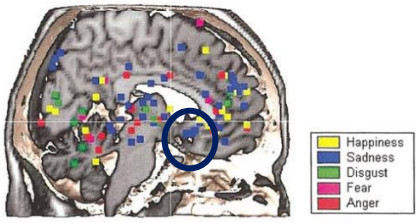
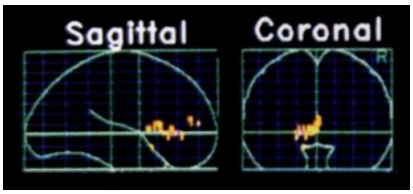
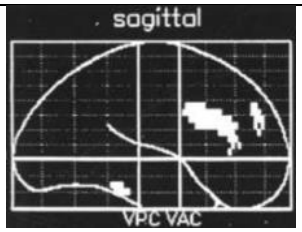
It is possible that the apparent mismatch in function of these vmPFC subregions in fear conditioning/extinction is because their functions are far more complex than has been originally proposed (Wallis et al., 2017), and with current assays we are unable to tease about the precise contributions of these regions. A focus on negative emotion clearly omits the important functions of these regions in positive emotion (Marquis et al., 2007), instrumental responding (Sharpe and Killcross, 2015a, 2015b) and social cognition (Rudebeck et al., 2006). Indeed, given the myriad of roles that vmPFC sectors are involved in, some have suggested that these regions have functions transcending isolated behaviours measured by specific tasks (e.g. in conditioned fear/extinction). For instance, Sharpe and Killcross have suggested that PL is involved in selective attention to aspects of the environment that are best predictive of an outcome (Sharpe and Killcross, 2014). Furthermore, the link between the role of IL to modulate extinction on the one hand (see **1.2.3.1.1**), and to attenuate goal-directed behaviour to promote habit formation on the other (Haddon and Killcross, 2011), still needs to be resolved. Further work is needed to determine the precise roles of IL-sgACC/25 and PL-pgACC/32, and to delineate their contributions to behaviour.

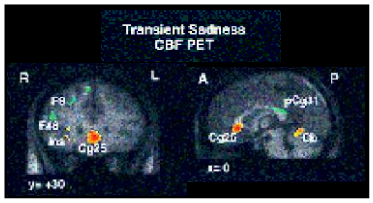
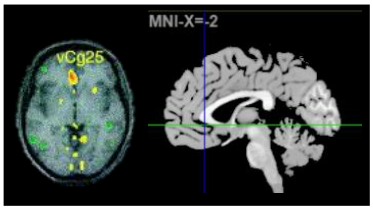
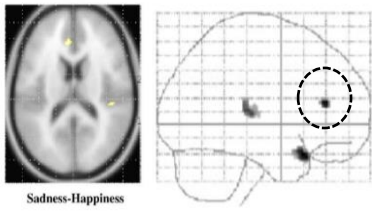
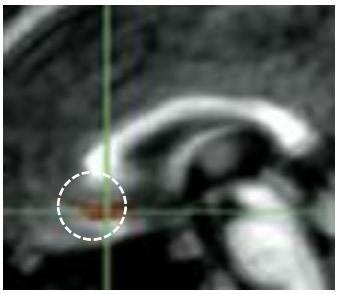
1.2.4 Ventromedial prefrontal cortex in the regulation of negative emotion: evidence from humans

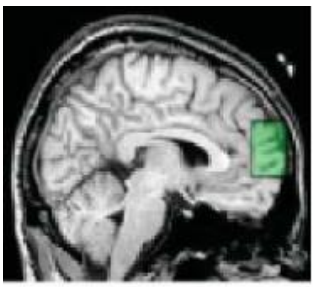
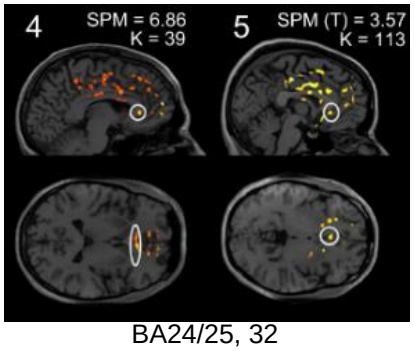
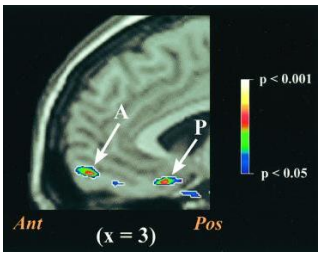
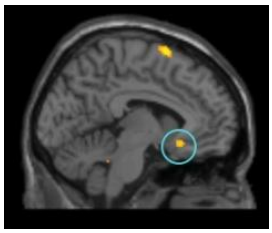
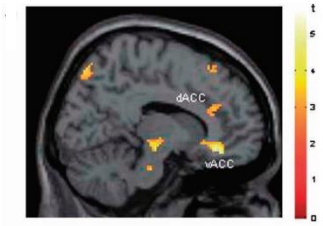
Human vmPFC has been implicated in three different aspects of negative emotion: (i) the feeling of sadness; (ii) sustained and unpredictable threat associated with anxiety; and (iii) top-down emotion regulation of fear and pain responses (Etkin et al., 2011).

1.2.4.1 Human vmPFC activity during the perception and expression sadness

Correlative human neuroimaging studies – including PET and fMRI – have given us insights into the role of the vmPFC in the perception and expression of sadness in healthy individuals. A meta-analysis of 55 neuroimaging studies found that sgACC/25 ‘activation’ (as measured by either an increase in rCBF or in BOLD signal) was significantly associated with sadness induction (46% of studies included) (Phan et al., 2002). Several studies illustrating vmPFC activation associated with negative mood induction are illustrated in **TABLE 1-3**.

Reference	Image	Description
	Meta-analyses of emotion activation studies	
(Phan et al., 2002)	 Circled: sadness-associated activation/ increase in rCBF focus in sgACC/25 (46% of studies)	Meta-analysis: Review 55 PET and fMRI activation studies. Sadness induction in healthy subjects significantly associated with 'subcallosal cingulate' (sgACC/25) activation/increase in rCBF. Overall, 46% of studies reporting this finding. Inconsistencies may be explained by differences in provocation method. Several early studies scanned participants whilst they were actively generating the desired emotional state (e.g. (Gemar et al., 1996; George et al., 1995; Pardo et al., 1993)). Studies which scanned subjects once they had generated the desired state (Liotti et al., 2000; Mayberg et al., 1999) yielded robust activation within sgACC/25.
	Recall of sad life events and autobiographical scripts	
(George et al., 1995)	 Bilateral ACC: BA24,32 Inferomedial PFC: BA25	rCBF PET, recall of sad life events: Increased rCBF to bilateral ACC and 'inferomedial prefrontal cortex' (Talairach coordinates [-14,8,-8] correspond to sgACC/25) associated with recall of sad memories *vs.* neutral memories.
(Gemar et al., 1996)	 BA24, 32	rCBF PET, recall of sad life events: Decreased rCBF to 'left medial prefrontal' cortex (dACC/24 and pgACC/32) associated with recall of negative life events.

(Mayberg et al., 1999)	 BA24, 25, 32	rCBF PET, autobiographical scripts: Subjects allowed 8-10 minutes to achieve target mood, mood state maintained for 2 minutes then tracer injected for scan. High levels of sadness (75% of subjects reported being 'tearful') associated with increased rCBF in sgACC/25 and anterior insula.
(Liotti et al., 2000)	 BA24, 25	rCBF PET, autobiographical scripts: Sadness-related effects included paralimbic activation in sgACC/25 and right posterior/left anterior insular cortex. These changes were not seen during anxiety-induction.
(Habel et al., 2005)	 BA24/32	fMRI, facial expressions + recall of sad life events: Increased activity in the d/pgACC (BA24/32) demonstrated when subjects viewed sad *vs.* happy faces, then instructed to use these facial expressions as material to generate their own emotional experience from recollection of sad events.
Impersonal sad stimuli		
(Paradiso et al., 2003)	 BA14, 25	rCBF, sad pictures: elevated blood flow to vmPFC during the processing of sad visual stimuli (compared to either neutral or happy stimuli).

(Côté et al., 2007)	 BA10	fMRI, film stimuli: compared to neutral film excerpts, sad film excerpts cause activation of very rostral vmPFC (BA10) together with frontopolar cortex.
(Smith et al., 2011)	 BA24/25, 32 [fMRI foci from two of the nine subjects; sgACC/25 seed circled]	fMRI, sad pictures and mournful music: sadness-induction paradigm combined with participant self-report when desired mood state was achieved. Activation within sgACC/25 was associated with successful attainment of a sad mood state, but inter-individual variability meant the specific locus varied.
(Zald et al., 2002)	 [P = Posterior focus] BA 25	rCBF, self-rating of negative affect: Individuals who report higher negative affect across the previous month show elevated blood flow to a posterior region of vmPFC corresponding to sgACC/25.
	<u>Social exclusion</u>	
(Masten et al., 2009)	 BA25	fMRI, social exclusion: greater activity in sgACC/25 relates to increased distress during social exclusion.
(Onoda et al., 2009)	 BA14, 25	fMRI, social exclusion: greater activity in vmPFC (sgACC/14,25) associated with increased social pain (low self-esteem, low belongingness, high meaningfulness and low control) during social exclusion.

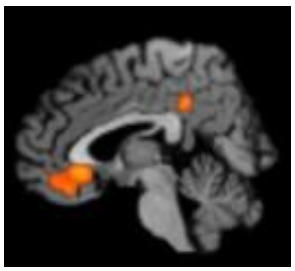
(Vijayakumar et al., 2017)	 BA14, BA25	Meta-analysis, social exclusion: bilateral vmPFC involvement across different paradigms used to measure feelings of social exclusion. vmPFC activation was strongest in adult samples, whereas ventrolateral involvement was more prevalent in developmental samples.

Table 1-3 Human vmPFC activations related to sadness and social exclusion. Phan *et al.*: meta-analysis. Other studies grouped according to elicitation method: (i) recall of sad life events with and without autobiographical scripts; (ii) impersonal sad stimuli (e.g. sad pictures); and (iii) social exclusion. Consistent with the findings of the meta-analysis, these studies implicate a caudal subregion of vmPFC, including but not limited to sgACC/25, in the experience of sadness across different elicitation methods.

Neuroimaging studies which have investigated the neurobiological basis of sadness must be interpreted with some degree of caution. Firstly, the precise method of emotion induction may influence patterns of rCBF/BOLD activity changes. For example, Reiman *et al*. found increased caudal vmPFC activity to recall-generated but not film-induced sadness, suggesting that such activity may relate to "*the cognitive process of internally generating emotion"* rather than the experience of sadness per-se (Phan et al., 2002; Reiman et al., 1997). Indeed, in the 2002 meta-analysis, many sgACC/25 activations arose from studies where autobiographical scripts were used to induce sadness (George et al., 1995; Lane et al., 1997; Liotti et al., 2000; Mayberg et al., 1999) although statistical analysis did not support the notion that sgACC/25 activation was specifically associated with recall. Secondly, the time course of scanning in relation to mood induction is important. Several early studies showed inconsistent reports of sgACC/25 activation (such as (Gemar et al., 1996; Pardo et al., 1993)) – in these studies, subjects were imaged (partly or fully) whilst they were *generating* the emotional state, such as whilst they were visualising emotional memories. rCBF-PET imaging experiments by Mayberg and colleagues (Liotti et al., 2000; Mayberg et al., 1999) addressed these issues by scanning only when the subjects had *achieved* the desired emotional state (and detected highly consistent increases in rCBF in sgACC/25). Finally, there are practical issues associated with fMRI of caudal vmPFC at high field strength owing to signal drop out (Wang et al., 2005). This measurement bias can mean that sgACC/25 is erroneously excluded in task-based and resting state fMRI studies.

1.2.4.2 Human vmPFC activity during sustained and unpredictable threat associated with anxiety

The neural circuitry associated with sustained threatening states is poorly understood. Such states are associated with heightened vigilance, future-oriented cognitive processing and representation of potential danger. Several studies have shown that these states engage the caudal vmPFC. For example, Hasler and colleagues have found that sgACC/25 and hippocampus show increased rCBF during sustained unpredictable threat (Hasler et al., 2007a). In addition to playing a role in monitoring – responding to salient events in the environment (Berns et al., 2001) – sgACC/25 is highly interconnected to regions involved in the behavioural, autonomic and endocrine responses to aversive threat such as the amygdala, hypothalamus and PAG (Drevets et al., 1998). These characteristics position sgACC/25 as a region well-suited to coordinate such these responses when encountering threats. This finding has been replicated in subsequent studies such as (Alvarez et al., 2011), which found transient increases in sgACC/25 activity to the onset of unpredictable threat, followed by sustained decreases. Alvarez and colleagues posit that the sustained decrease may reflect changes in "*the regulation of visceral reactions during threat exposure*."

The clinical neurobiology of fear and anxiety can also be probed using specific pharmacological agents to induce such states in healthy individuals during functional imaging (Shin and Liberzon, 2010). Cholecystokinin-4 administration is associated with increased subjective fear and anxiety, along with increased activation of vmPFC (primarily BA10) (Eser et al.; Javanmard et al., 1999). Administration of procaine also induces elevated fear and anxiety, together with increases in rCBF to the pgACC (Ketter et al., 1996). A subsequent study supported the finding of increased pgACC activation associated with procaine-induced negative emotion, but observed that subjects who did *not* have a panic attack evidenced greater pgACC activation compared to those who did (Servan-Schreiber et al., 1998) supporting a top-down regulatory role for this region. Finally, the α_2-adrenoreceptor agonist yohimbine has been associated with increased subjective anxiety together with increased rCBF to the vmPFC (BA10) (Cameron et al., 2000).

1.2.4.3 Human vmPFC activity in the top-down regulation of negative emotion

Largely grounded in pre-clinical work in animals (see sections **1.2.3.1.1** and **1.2.3.1.3**), human vmPFC has been implicated in the top-down regulation of emotion. Across several paradigms, vmPFC activity has been shown to influence activity within subcortical structures critical in emotion generation including the amygdala (Etkin et al., 2006; Meyer-Lindenberg and Zink, 2007). The role of human vmPFC in top-down regulation is apparent in lines of work linking it to fear extinction; stress controllability and adaptation; fear generalisation and pain expectation/placebo effects.

1.2.4.3.1 Fear extinction

Several studies have linked structural (Milad et al., 2005) and functional (Milad et al., 2007a; Phelps et al., 2004; Schiller et al., 2008) characteristics of human vmPFC to fear extinction – two key examples are shown in **FIGURE 1-11**. Initial work by Elizabeth Phelps *et al*. utilised a fear acquisition/extinction/extinction-recall paradigm similar in design to that used in rodents, and found that *depressions* in activity of a rostral portion of sgACC (BA10/11) *diminished* as extinction learning progressed (as measured by skin conductance responses) (Phelps et al., 2004). The most striking correlation was measured at extinction recall – subjects showing greater extinction on extinction days showed reduced rostral sgACC depression at the start of extinction recall, highlighting a potential role of this region in the retention of extinction. Furthermore, on extinction recall days alone, responses of rostral sgACC were correlated with the strength of amygdala responses, suggesting that vmPFC activity is linked to top-down regulation of amygdala responses specifically during the retention of extinction.

A subsequent study broadly replicated these findings. Milad and colleagues again observed diminished activity of a more caudal sgACC region (sgACC/25 and BA10) associated with differential fear conditioning during acquisition, which diminished as extinction learning progressed (Milad et al., 2007a). In this study, a more 'direct' correlation between caudal sgACC signal change and extinction recall was observed: on the recall day itself, activations in caudal sgACC directly correlated with the extinction recall (measured by skin conductance responses over the first four trials). However, it is worth noting that whilst Phelps *et al.* observed diminished sgACC activity throughout all phases, Milad and colleagues observed moderate sgACC *activation* correlated with extinction recall (more consistent with rodent work demonstrating increased IL neuron responses correlated with extinction recall) (Milad and Quirk, 2002). The reasons for this difference are not clear, although in Phelps *et al*., extinction recall was compromised by high levels of fear at test which meant the first three recall trials had to be removed, potentially influencing the magnitude of vmPFC signal change measured across the session.

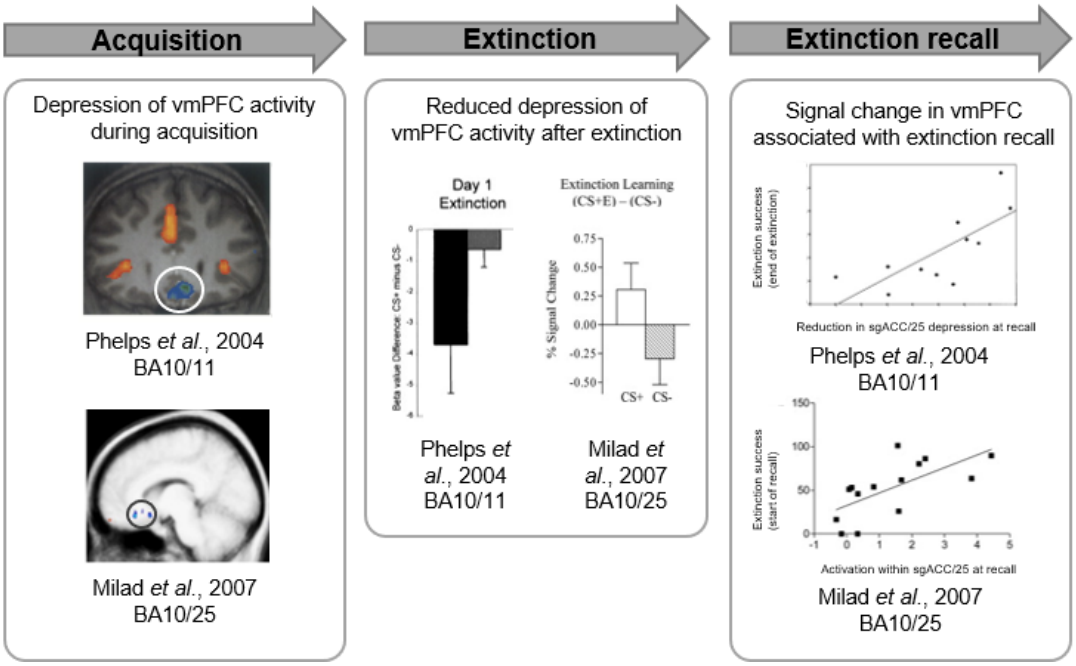


Figure 1-11 Neuroimaging studies implicating human vmPFC in fear extinction. Both Phelps *et al.* (rostral sgACC, BA10/11) and Milad *et al.* (caudal sgACC, BA10/25) found activity in vmPFC decreased during fear acquisition. During extinction, both studies observed diminished depression (i.e. a positive activity change) in vmPFC by the end of extinction. On the following day during extinction recall, different effects are reported in the two studies. Phelps and colleagues found that diminished depression in rostral sgACC activity at the start of extinction recall was correlated with extinction success on the previous day. Evidencing a subtly different effect, Milad and colleagues showed caudal sgACC *activation* correlated with the degree of extinction retention measured on the same day. Despite these differences, both studies implicate regions of vmPFC in the recall of extinction

1.2.4.3.2 Controllability of stress and stress adaptation

vmPFC activity has been linked to controllability of aversive stimuli, with fMRI studies showing that the vmPFC is engaged in a myriad of situations involving stress adaptation and control. These include during situations of increased perception of control and persistence during uncontrollable setbacks (Bhanji and Delgado, 2014); during periods of active coping (Sinha et al., 2016); during negative affect reduction in response to picture stimuli (correlated with steeper declines in cortisol levels) (Urry et al., 2006); and during the perception of control over painful stimuli (Salomons et al., 2004; Wiech et al., 2006).

Recent work by Kerr and colleagues has posited that the vmPFC is particularly important in generating *preparatory* emotional responses when subjects *anticipate* having control over outcomes. The perception of control during anticipation of aversive stimuli is associated with robust engagement of human vmPFC (BA10/14), whereas vmPFC is not activated in

situations where aversive stimulus presentation is anticipated as being uncontrollable (Kerr et al., 2012). Furthermore, during anticipation of controllable threat, human vmPFC shows strong functional coupling with the amygdala (Kerr et al., 2012) – supporting a role in preparatory top-down regulation.

1.2.4.3.3 Fear generalisation

Human functional neuroimaging studies have supported a role for vmPFC in the transfer of conditioned fear to stimuli perceptually similar to learned CSs. Activation of the vmPFC (including sgACC/25 and BA10) and insula increases as generalisation stimuli become more distinct from the CS, correlated with participants' decreasing ratings of shock likelihood and pupillary responses (Greenberg et al., 2013). This generalisation effect was replicated in a second study, although the region of vmPFC whose activity varied with perceptual similarity was more rostral (BA10, 32) (Lissek et al., 2014). Increased activation of vmPFC as stimuli become more distinguishable may be interpreted as increased safety signalling and fear inhibition, again consistent with work linking human vmPFC to the top-down regulation of emotion.

1.2.4.3.4 Pain and placebo effects

An additional line of evidence implicating human vmPFC in top-down regulation of emotion derives from its involvement in mediating placebo effects. A recent meta-analysis showed increases in rostral vmPFC (BA10) activity associated with placebo effects and expectancy manipulations to reduce pain (Atlas and Wager, 2014). Isolated studies have also shown that treatment with placebo reduces activity in pgACC/32 (Eippert et al., 2009).

1.2.5 Ventromedial prefrontal cortex in social cognition

The role of the vmPFC in social cognition is likely an emergent property of its involvement in positive and negative affect. Nevertheless, a large body of work has emerged specifically implicating vmPFC function in social processing (Hiser and Koenigs, 2018). In particular, extensive evidence comes from patients with vmPFC lesions, who show impairments in various aspects of social processing: cognitive empathy (Barrash et al., 2000; Shamay-Tsoory et al., 2009); distinguishing emotional expressions on faces (Heberlein et al., 2008; Tsuchida and Fellows, 2012); attending to eye regions of faces (Adolphs et al., 2005; Wolf et al., 2014, 2016); and in moral judgement (Fumagalli and Priori, 2012; Young and Koenigs, 2007). The vmPFC is engaged robustly when recalling autobiographical memories – and in this role, interacts with the default mode network (DMN), including the dmPFC (Northoff et al., 2006; Raichle et al., 2001; Svoboda et al., 2006).

1.3 Physiological Function and the Ventromedial Prefrontal Cortex

The anatomical connections of vmPFC place it at the interface between cognition and emotion, important in the detection of external and internal challenges to homeostasis together with coordinating the autonomic and endocrine effector mechanisms to maintain a stable internal milieu (Joyce and Barbas, 2018). The role of the vmPFC in the regulation of physiological function – in particular, regulation of the autonomic nervous system and in the regulation of endocrine axes – can be studied either in emotionally-neutral conditions (e.g. quite awake or anaesthetised) or during conditions of concurrent emotion regulation (e.g. periods of stress).

Several considerations must be borne in mind when investigating cortical regulation of autonomic and cardiovascular function:

- **Which species is being investigated?** Beyond issues of anatomical homology between rodents, NHPs and humans, there are apparent functional differences between putatively homologous regions in rodents and primates in cardiovascular control. For example, whilst electrical and chemical *stimulation* of rodent IL appears to have 'sympatho-inhibitory' effects (Al Maskati and Zbrożyna, 1989), pharmacological *inactivation* of the putative homologue in primates (sgACC/25) increases vagal tone (Wallis et al., 2017) meaning that opposite manipulations of putatively similar regions have comparable functional outcomes. Indeed, **Chapter 3** of this thesis shows that stimulation of marmoset sgACC/25 has reduces vagal tone and increases sympathetic:parasympathetic balance.
- **Is the preparation awake, or anaesthetised?** Many animal studies investigating the prefrontal regulation of cardiovascular function are carried in anaesthetised preparations. Anaesthesia is known to profoundly alter cardiovascular activity (Vatner, 1978) so the results of these studies must be interpreted with caution. Studies have sometimes found opposite effects of manipulating vmPFC on cardiovascular parameters in awake *vs*. anaesthetised animals (Burns and Wyss, 1985).
- **How was cortex manipulated?** In early electrical stimulation studies, the frequency of stimulation and pulse duration was important and could result in differing magnitudes of effects (Kaada et al., 1949). In addition, the nature of the effect of electrical stimulation on activity within a brain region is unclear – whether application of electrical current is analogous to 'activating' (or inhibiting) an area is not known, but it is appreciated that such a manipulation disrupts normal activity within the brain region targeted. It is also important to note that sufficiently large currents can activate adjacent fibre pathways (Loewy and Spyer, 1990).

- **Which region of cortex was manipulated?** As discussed in **1.1.2**, the vmPFC of both rodents and primates is heterogeneous and consists of subregions. The results of early studies can be difficult to compare as investigators did not localise their manipulations to recognised cytoarchitectonic boundaries (Loewy and Spyer, 1990).

1.3.1 Ventromedial prefrontal cortex regulates autonomic and cardiovascular function in emotionally-neutral situations

Although one must be mindful of the above caveats, a large body of work from studies in rodents, NHPs and humans supports the notion that vmPFC subregions are involved in the regulation of cardiovascular function in relatively emotionally-neutral situations. Note that studies involving non-emotional tasks concurrent with physiological monitoring (e.g. handgrip studies) will also be considered in this section.

1.3.1.1 Evidence from rodents

Early work examining the anatomical connectivity of rodent vmPFC subregions initially pointed to a role in autonomic function. A series of studies carried out in the 1980s by Edward Neafsey and Robert Terreberry injected the mixed anterograde-retrograde tracer wheat germ agglutinin–horseradish peroxidase into the dorsal medulla (corresponding to the nucleus of the solitary tract [nucleus tractus solitarius, NTS]/dorsal motor nucleus of the vagus nerve), and found retrograde labelling of cell bodies in the rat vmPFC: predominantly IL, with additional neurons in ventral PL ('PLv') (Terreberry and Neafsey, 1983). Additionally, injection of the tracer into vmPFC (AC, PL and IL) resulted in anterograde labelling of terminals in the NTS (**FIGURE 1-12A, B**). Given the direct connections of these vmPFC regions to autonomic effector regions in the brainstem, Terreberry and Neafsey collectively termed PLv and IL 'visceral motor cortex' (**FIGURE 1-12C**).

In the early 1990s, more detailed anatomical characterisation of vmPFC-brainstem connectivity corroborated this work. For example, Hurley *et al*. showed that efferents from IL terminated in boutons at preganglionic parasympathetic neurons in the dorsal motor nucleus of the vagus, together with preganglionic sympathetic neurons of the intermediolateral nucleus of thoracic spinal cord (Hurley et al., 1991). These pathways have been termed a 'visceral pyramidal tract system' (Neafsey et al., 1993). Retrograde tracing from the stellate ganglion (cervical sympathetic ganglion outputting to the heart) using pseudorabies virus showed extensive trans-neuronal labelling in IL, providing additional support for a role for IL in sympathetic modulation of cardiac function (Westerhaus and Loewy, 2001).

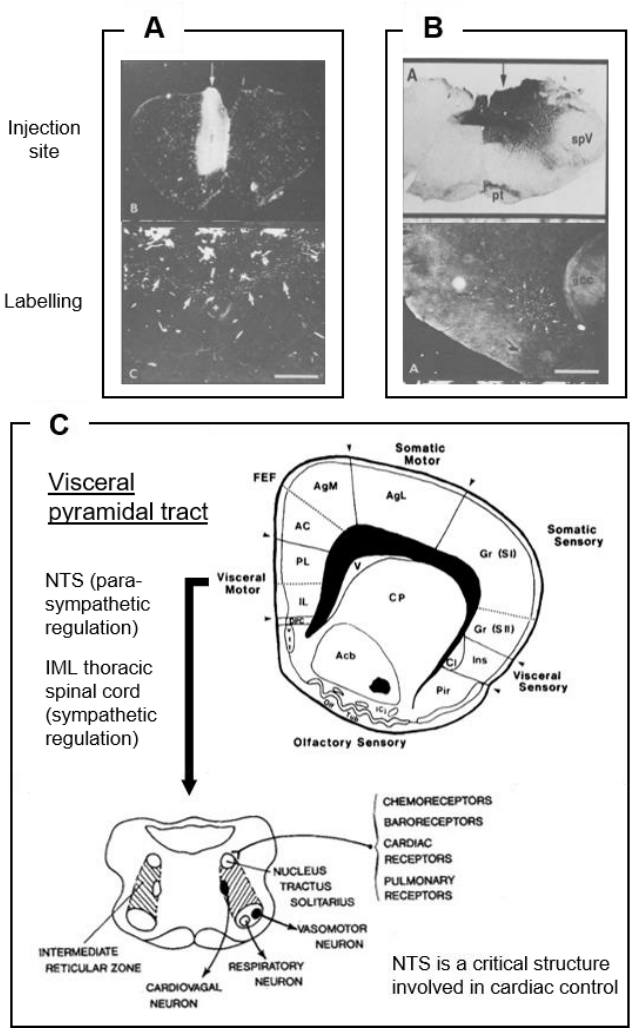


Figure 1-12 Bidirectional connectivity between rodent vmPFC and autonomic control centres in the brainstem. **A** and **B** taken from Terreberry and Neafsey, 1983; **C** adapted from Neafsey *et al*. in *Central Regulation of Autonomic Function* (eds. Loewy, Spyer; 1990). **A** Injection of the mixed anterograde-retrograde tracer wheat germ agglutinin–horseradish peroxidase into vmPFC (including PL, IL and AC; top) causes anterograde labelling of fibres in the NTS (bottom). **B** Injection of the tracer into the dorsal medulla (encompassing NTS; top) causes retrograde labelling of cell bodies in rodent lL and ventral PL'v' (bottom). **C** The direct connections between IL and PLv to brainstem structures and structures in the spinal cord led to the proposal that these two regions constitute visceral motor cortex, from which a visceral pyramidal tract originates. The NTS is shown, containing populations of neurons involved in HR regulation (cardiovagal neurons), together with regulation of peripheral vascular resistance (vasomotor neurons).

Whilst rodent IL/PL directly project to brainstem autonomic motor regions, they also project to other autonomic control regions which in turn project to the brainstem – namely, the hypothalamus, amygdala and insula (Loewy and Spyer, 1990; Vertes, 2004). A reasonable question, therefore, is whether direct or indirect projections from IL/PL to the brainstem

mediate their autonomic effector functions. Indeed, most descending vmPFC-NTS projections are not direct, but travel in the medial forebrain bundle to terminate in hypothalamic nuclei, which then coarse through dorsal brainstem to the medulla (Hurley et al., 1991). Supporting a role for indirect projections, hypotension generated by electrical stimulation of vmPFC is blocked with lidocaine infusions into either the lateral hypothalamus (LH) or PAG (Fisk and Wyss, 2000; Hardy and Holmes, 1988).

There are other studies, however, which emphasise the importance of direct PL/IL-brainstem projections. In one study which mapped cardiovascular correlates of electrical stimulation of sites within vmPFC of anaesthetised rats (Al Maskati and Zbrożyna, 1989), three types of site were identified: defensive inhibitory sites (predominantly PL), sympatho-inhibitory (predominantly IL) sites and ineffective sites (**FIGURE 1-13A**). Stimulation of sympatho-inhibitory sites resulted in baseline hypotension without any effect of hypertension induced by amygdala/hypothalamus stimulation (suggestive of direct pathways to the brainstem). Stimulation of dorsal defensive inhibitory sites had no effect on baseline cardiovascular parameters, but blocked hypertension and defensive reactions triggered by either amygdala or hypothalamic stimulation (**FIGURE 1-13B**). The authors highlight that the suprathreshold stimulation of the amygdala/hypothalamus utilised in the study would activate all neurons within the vicinity, suggesting that the action of defensive inhibitory sites must be independent of effects on these subcortical structures – instead, their effects are exerted at the brainstem/spinal cord (**FIGURE 1-13C**). It is likely that the influence of PL/IL on autonomic function reflects a combination of direct and indirect projections to the brainstem.

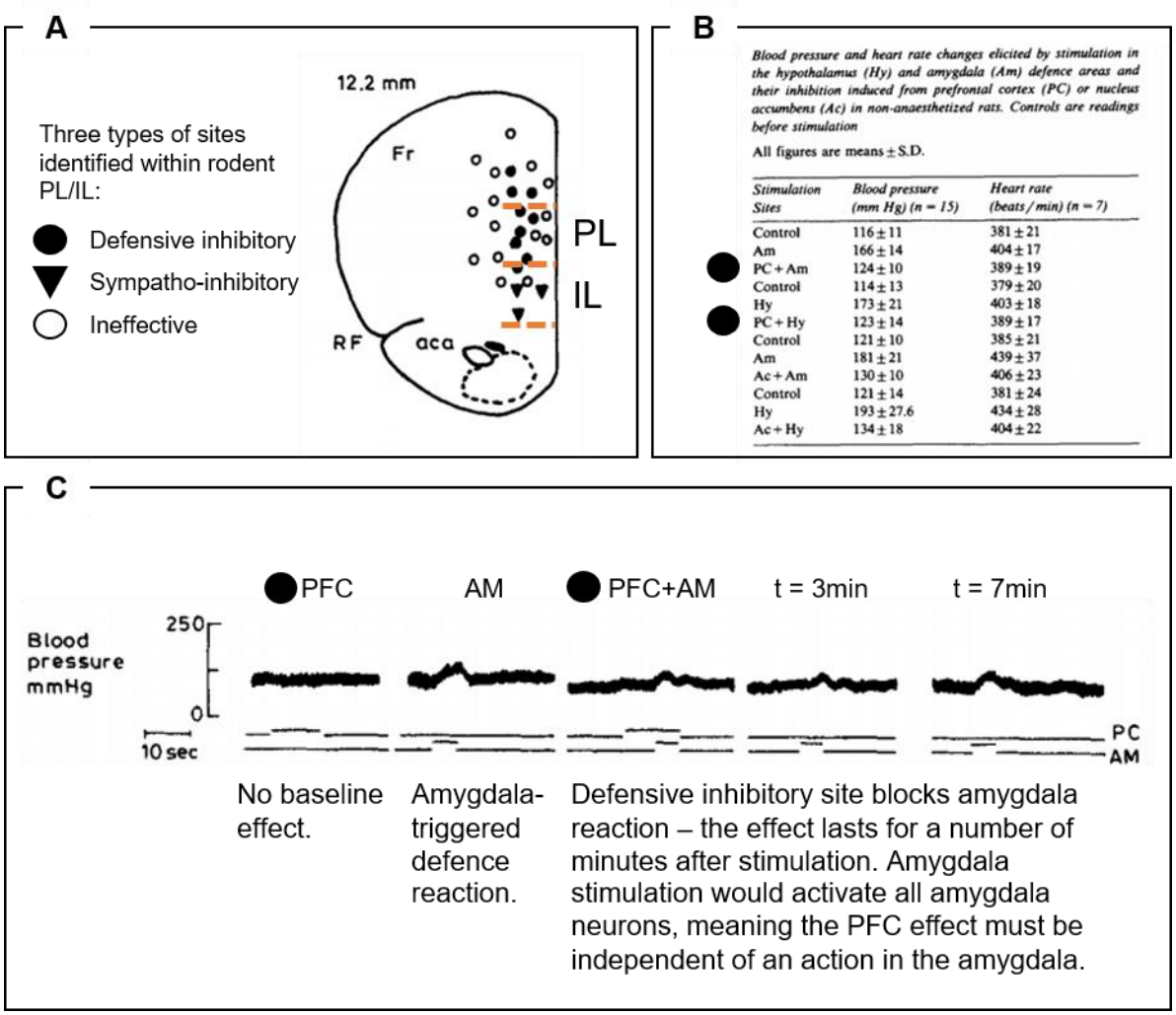


Figure 1-13 Autonomic effects of rodent vmPFC stimulation in the context of amygdala and hypothalamic stimulation. Figure adapted from Al Maskati and Zbrożyna, 1989. **A** Schematic diagram of rodent mPFC, showing the location of defensive inhibitory sites (PL), sympatho-inhibitory sites (IL) and ineffective sites. For a detailed description of the function of these sites, see text. **B** Table showing BP and HR changes associated with isolated amygdala or hypothalamic stimulation, together with combined defensive inhibitory stimulation (marked with a black circle). **C** BP trace from an example animal. Stimulation of defensive inhibitory sites (far left) has no baseline effect whereas stimulation of the amygdala (AM) induces a 'defensive reaction' including hypertension. Combined defensive inhibitory and amygdala stimulation (PFC+AM) blocks the defensive reaction; the effects of a single 10s stimulation lasts for several minutes afterwards (right). Given that amygdala stimulation would activate all neurons in its subnuclei, the antagonistic effects of defensive inhibitory stimulation *must* be mediated by effects downstream of the amygdala (in the brainstem).

Following on from these early studies, increasing evidence supports the idea that rodent vmPFC (especially IL) functions as visceral motor cortex. As the function of these brain regions is being studied at finer and finer resolution, so discrete sites have been identified

within the vmPFC which exert control over specific aspects of autonomic function including blood pressure (BP), heart rate (HR) and baroreceptor gain (Cechetto, 2014; Resstel and Corrêa, 2006a):

- **vmPFC effects on BP:** Direct stimulation of vmPFC (encompassing both PL and IL) causes an evoked mean arterial pressure (MAP) decrease in anaesthetised rats accompanied by increased blood flow in iliac vasculature (Owens and Verberne, 2001). Acetylcholine injections into IL also cause hypotension and peripheral vasodilation (Crippa et al., 2000). Several lines of evidence do, however, point to confounding effects of anaesthesia: in un-anaesthetised rats, vmPFC stimulation by electrical (Burns and Wyss, 1985; Tavares et al., 2004) and chemical (Resstel and Corrêa, 2005) methods causes an opposite pattern of hypertensive and tachycardic changes.
- **vmPFC effects on baroreflex responses:** Excitotoxic lesions of the entire rodent vmPFC (including IL, PL and portions of AC) reduce sensitivity of the baroreceptor HR reflex (*i.e.* per unit change in BP, the HR change is less). Pharmacological manipulations of IL can also reduce baroreflex sensitivity (e.g. NMDA receptor antagonism) (Resstel and Corrêa, 2006b).
- **vmPFC sympatho-inhibitory effects:** Work in rodents points to a sympatho-inhibitory default mechanism within IL (Shoemaker and Goswami, 2015). For example, electrical stimulation of IL has inhibitory effects within the medulla (at latencies consistent with a monosynaptic pathway) and reduces sympathetic splanchnic nerve discharge (with a latencies consistent with a polysynaptic pathway) (Verberne, 1996).
- **Effects of chronic *vs.* acute vmPFC manipulations**: Chronic lesions to rodent vmPFC have no impact on baseline BP or HR (Verberne et al., 1987), suggesting that rodent vmPFC exerts little tonic influence on the cardiovascular system. However, these large lesions may be affecting multiple subregions with potentially different (even opposite) roles in cardiovascular function.

1.3.1.2 Evidence from NHPs

Anatomical tracing studies in NHPs also support a role for vmPFC in autonomic regulation – neurons from vmPFC (sgACC/25) have “massive” projections to hypothalamic autonomic nuclei which then project to NTS and spinal autonomic centres (Barbas et al., 2003). The vmPFC also diffusely innervates multiple amygdala nuclei, meaning it has dual access to an emotional-visceral motor system (Alheid and Heimer, 1996; Holstege, 1991). Several other tracing studies have supported the notion that subregions of NHP vmPFC are linked to

autonomic effector regions and autonomic output (An et al., 1998; Chiba et al., 2001; Ghashghaei and Barbas, 2002; Ongür et al., 1998a; Rempel-Clower and Barbas, 1998).

More recently, anatomical tracing work in the macaque has shown that affective areas of cerebral cortex – including sgACC/25, pgACC/32 and dACC/24 – project to the adrenal medulla. Dum *et al.* injected rabies virus into the adrenal medulla and used a survival-time analysis method to identify third and fourth order neurons in the prefrontal cortex which contribute to a 'cortico-adrenal circuit' (Dum et al., 2016). They found prefrontal components of a 'medial network' (vmPFC – BA25, 32 and 24c) are the source of the densest projections the adrenal medulla. These regions are broadly similar to regions identified in human functional imaging studies related to sympathetic-related activations, negative affect and cognitive control. Such circuits may, therefore, mediate the effects of internal states such as chronic stress on visceral function.

Early functional work – much of which was carried out in macaques – largely focused on determining the contributions of the cingulate gyrus to autonomic regulation, rather than the involvement of the vmPFC specifically. In 1945, an 'electrically responsive rostral cingulate field' was identified in BA24 of anaesthetised macaques (Smith, 1945). Depending on the precise stimulation parameters, application of a current to this zone resulted in varied yet profound changes in HR and BP. In the same study, severing the vagus nerve abolished the cardiac effects, suggesting that the effects of rostral cingulate stimulation are mediated through changes in vagal tone. Similar results were obtained by other investigators using electrical stimulation techniques in anaesthetised macaques (Ward, 1948), although stimulation in awake animals has yielded more variable results (Anand and Dua, 1956).

Evidence for a role of ventral subregions came in 1949, when Kaada and colleagues applied electrical stimulation to pgACC and sgACC in anaesthetised macaques (Kaada et al., 1949). Stimulation throughout these regions induced cardiovascular changes, but the most prominent cardiovascular change was observed in 'posterior subcallosal cortex,' corresponding to sgACC/25. In addition to having a respiratory effect, application of electrical current in this region produced a BP response characterised by a transient hypertension followed by a more prolonged – but still short-lived – refractory hypotension (**FIGURE 1-14**). Since this study, little work has been carried out investigating the role of primate vmPFC in autonomic regulation, beyond the observation of enhanced activity within sgACC/25 during vegetative states such as sleeping, potentially reflecting an influence on parasympathetic activity (Rolls et al., 2003b).

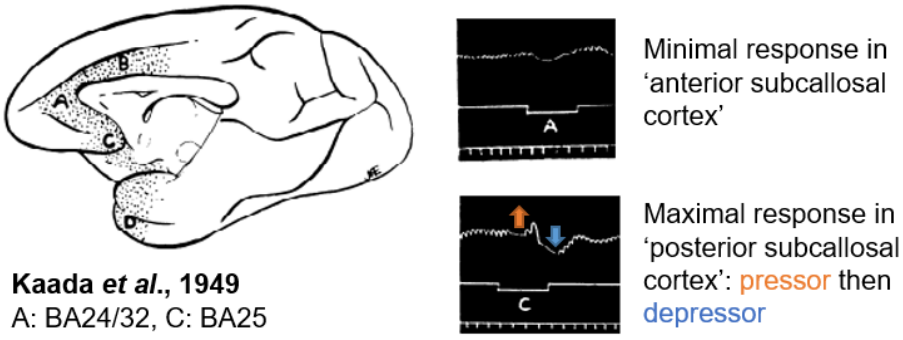
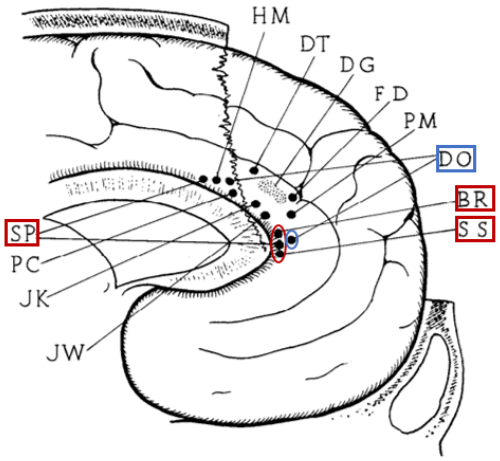


Figure 1-14 Electrical stimulation of pgACC and sgACC of the macaque induces autonomic changes. Figure adapted from Kaada *et al*., 1949. Stimulation of the 'anterior subcallosal cortex' (pgACC/32, marked A) causes minimal autonomic change. Stimulation of the 'posterior subcallosal cortex' (sgACC/25, marked C) induces transient hypertension followed by a more prolonged (but also short-lived) hypotension.

[Work described in this paragraph was published in *Proceedings of the National Academy of Sciences, USA* in 2017 with the present author as a co-author: (Wallis et al., 2017)] The effects of targeted pharmacological manipulations within NHP vmPFC on autonomic parameters during an emotionally neutral, quiet resting state have only very recently been explored using marmosets. Wallis and colleagues compared the effects of inactivating sgACC/25 and pgACC/32 on BP, HR and vagal/sympathetic balance in neutral conditions (Wallis et al., 2017). Inactivation of sgACC/25 was found to have profound effects on cardiovascular activity, reducing HR and BP and increasing heart rate variability (HRV). When the effects on HRV were fractionated into vagal and sympathetic contributions, sgACC/25 inactivation had a selective effect to increase cardiac vagal tone. By contrast, inactivation of pgACC/32 had restricted effects on cardiovascular responses in emotionally neutral conditions limited to a modest increase in BP. This suggests that NHP sgACC/25 has a critical causal role in establishing activity within a central autonomic network, modulating the balance between parasympathetic and sympathetic contributions to cardiac function.

1.3.1.3 Evidence from humans

In a similar vein to work in macaques at the time, electrical stimulation experiments have been carried out in humans assessing cardiorespiratory changes induced by stimulating portions of the cingulate gyrus, including pgACC/32. Pool and Ransohoff stimulated the rostral cingulate (including pgACC) in twelve anaesthetised patients during neurological surgery (Pool and Ransohoff, 1949). Although the effects were variable in magnitude and direction, stimulation throughout the bilateral rostral cingulate zone (BA24, 32) resulted in BP and HR changes (**FIGURE 1-15**), whereas stimulation of more superior dmPFC (BA9/10) had no effect.



Pool and Ransohoff, 1949

pgACC: BA24 and BA32

Patient	Blood pressure (sys/dia)	Heart rate
DO	Increase 60/30	Increase 12bpm
BR	No effect	No effect
SS	Increase 15/10	No effect
SP	Increase 20/50	No effect

Figure 1-15 Electrical stimulation of human pgACC regions induces autonomic changes. Figure adapted from Pool and Ransohoff, 1949. Stimulation of the perigenual region induces variable autonomic changes. In patient DO (blue) stimulation of pgACC/32 profoundly increased systolic and diastolic BP and induced a moderate tachycardia. In patients BR, SS and SP, stimulation of pgACC/24 typically increased BP with no effect on HR.

The cardiovascular effects of direct electrical stimulation of human vmPFC have also been observed in the context of deep brain stimulation (DBS) for conditions such as depression and epilepsy. A recent study directly examined the cardiovascular consequences of DBS into sgACC/25 in patients undergoing electrode implantation as a prelude to surgery to relieve epilepsy (Lacuey et al., 2018). In the four patients where electrodes were placed in sgACC/25, stimulation produced consistent and striking hypotensive changes – specifically, a reduction in systolic BP with more variable changes in diastolic BP. Laucey and colleagues present precise electrode placement diagrams in three of the four patients, and it is worth noting that in two patients with more caudal electrode placements (within sgACC/25) the hypotensive effects are substantially greater than in the patient with a more rostral placement (strictly speaking within sgACC/14), although this interpretation is also confounded by differences in the laterality of the hemisphere stimulated (**FIGURE 1-16**). These data support earlier work in macaques, showing more substantial BP changes with stimulation of caudal sgACC/25 (Kaada et al., 1949). However, whether the neurophysiological consequence of DBS is one of stimulation, inhibition, or generalised disruption remains to be determined (Chiken and Nambu, 2014). Whilst DBS experiments such as that described in Laucey *et al.* point to a role of sgACC/25 in autonomic regulation, the precise nature of its role in humans is still unclear.

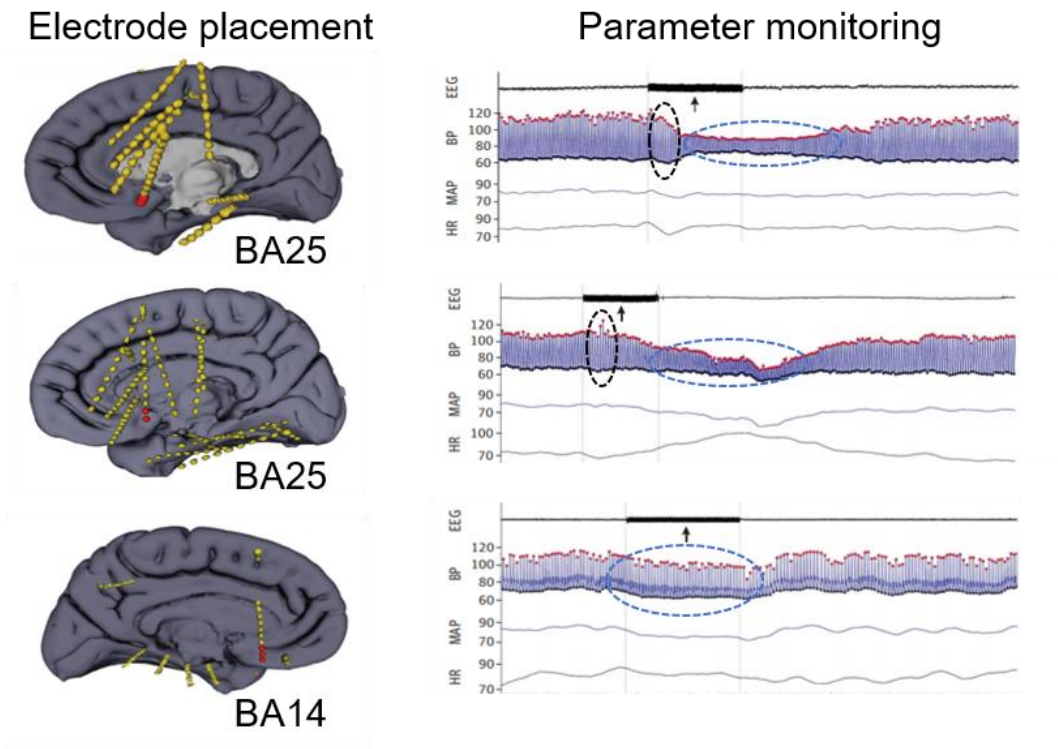


Figure 1-16 Electrical stimulation of human vmPFC induces BP and HR changes.
Figure adapted from Laucey et al., 2018. In this study, four patients were undergoing electrode implantation prior to epilepsy surgery. Stimulation of a subgenual region in produced hypotensive changes in all patients. The electrode placements of three patients (those available from the manuscript) are shown left. Right are the individual EEG, BP, MAP and HR traces. The time of stimulation is identifiable by a thick shading on the EEG (the midpoint is indicated by an arrow). In the upper two patients, stimulation produced hypertension at onset (black dashed oval). In all patients there was transient hypotension associated with stimulation (either during or after stimulation ended, blue dashed oval). Note that the bottom patient with (i) a more rostral placement (sgACC/14 rather than sgACC/25) and (ii) an electrode in the left rather than right vmPFC had the smallest cardiovascular response.

In addition to direct stimulation experiments, functional imaging data supports a role for vmPFC in cardiovascular control in conscious humans:

- **vmPFC activity associated with 'at-rest' cardiovascular modulation**: Whilst chronic lesion experiments in rodents suggest that vmPFC is not involved in regulating baseline cardiovascular parameters, in humans this may not be the case. Human vmPFC forms part of the DMN – a 'specific, anatomically defined brain system preferentially active when individuals are not focused on the external environment' (Buckner et al., 2008; Raichle et al., 2001). Default mode activity within vmPFC has been suggested to reflect regulation of parasympathetic outflow (vagal

tone) to maintain low baseline HR and high baseline HRV, constituting a key component of the 'cortical autonomic network' (Shoemaker and Goswami, 2015; Thayer et al., 2012). Indeed, vmPFC BOLD activity patterns have been directly correlated with high frequency band components of HRV, which is thought to reflect parasympathetic activity (Goswami et al., 2011).

- **vmPFC activity during volitional exercise:** vmPFC (primarily pgACC/24 and 32) has been recognised to form a key part of a cortical autonomic network based on a meta-analysis of cortical activations during non-fatiguing handgrip exercise (Shoemaker and Goswami, 2015; Shoemaker et al., 2015). Specifically, observations of reduced vmPFC activity have been consistently reported in volitional exercise tasks that involve a HR response (Goswami et al., 2011; Norton et al., 2013). Reductions in vmPFC activity have been interpreted as a removal of a 'parasympathetic break' to facilitate increases in HR during exercise (Shoemaker and Goswami, 2015).
- **vmPFC activity and baroreflex responses**: Baroreflex responses can be assessed in various ways – for example, volitional changes using Valsalva's manoeuvre (forceful exhalation against a closed airway); changes in the absence of volitional effort with lower-body suction (applying suction pressure to a supine body below the iliac crest); and assessment of spontaneous fluctuations in HR and BP. For example, King *et al.* showed elevated mPFC activity during the 'recovery' phase of the Valsalva manoeuvre, where BP is rising together with a reflex-induced cardioinhibitory effect (reductions in HR and sympatho-inhibition) (King et al., 1999)[1]. In general, regions corresponding to dmPFC/8,9 and dACC/24 – rather than vmPFC – have been more consistently implicated in baroreflex control, raising sympathetic drive by reducing baroreflex inhibition (Medford and Critchley, 2010). A comparable region has been identified related to sympathetic modulation of HR during cognitive and motor tasks (Critchley et al., 2003).

In sum, activity within vmPFC and dmPFC/dACC subregions of human PFC correlates with various aspects of cardiovascular function. The precise contributions these areas make is still not understood, although some broad inferences can be drawn. Caudal vmPFC activity seems to be related to parasympathetic outflow during 'at rest' conditions, whereas decreases in pgACC/32 activity are related to a release of inhibition over sympathetic outflow. Furthermore, increases in dACC activity are consistently linked to increases in sympathetic outflow. The role of vmPFC (and dACC) subregions in the regulation of autonomic function, together with their apparent extensive connectivity to

[1]It is not possible to determine the precise location of this mPFC activation from the manuscript as no co-ordinates are provided.

brainstem/subcortical structures involved in visceral function, has led to the suggestion that vmPFC subregions form core components of a 'central autonomic network' (CAN) in rodents, NHPs and humans (Loewy and Spyer, 1990). The CAN is proposed to be an integral regulatory system involved in visceromotor and neuroendocrine control essential for adaptation and survival.

1.3.2 Ventromedial prefrontal cortex regulates autonomic function during emotionally-valanced situations

This section will discuss vmPFC activity in relation to autonomic modulation (with a focus on cardiovascular function) during emotional situations, including situations of 'mental stress' (such as the pressured performance of Stroop tasks or mental arithmetic in humans) (Critchley et al., 2005; Mathias and Bannister, 1999).

1.3.2.1 Evidence from rodents

Several lines of work support a role for rodent vmPFC in stress-associated cardiovascular regulation, with differential roles for PL and IL emerging (Myers, 2017). Cobalt chloride injection (which silence inputs and outputs) into PL enhances tachycardia associated with restraint (but does not alter BP), whereas the same manipulation in IL reduces tachycardia (again, with no effect on BP) (Tavares et al., 2009). However the pattern of results from the literature is mixed – other studies have found no effect following muscimol-induced inhibition of IL, whereas activation of IL decreases HR and BP responses to air puff stress (Müller-Ribeiro et al., 2012). A potential explanation for these contrasting results might relate to a stressor-specific function of these subregions.

Supporting a role for rodent vmPFC in *learned* cardiovascular responses associated with stressful outcomes, global cobalt chloride inhibition of PL and IL reduces HR and BP responses in a context previously associated with foot shocks (Resstel et al., 2006). Data supporting separable roles for PL and IL in learned cardiovascular stress responses comes from lesion work by Frysztak and Neafsey. Whilst global excitotoxic lesions of PL and IL decreased HR responses to a tone predicting shock, lesions restricted to PL increased tachycardic responses suggesting that the region has sympatho-inhibitory functions (Frysztak and Neafsey, 1994). By contrast, excitotoxic lesions of IL reduced sympathetic-mediated tachycardia, suggesting an opposing role of PL/IL subregions in the cardiovascular correlates of learned fear. Given the aforementioned role of rodent vmPFC in the top-down regulation of emotion, together with evidence supporting the role of amygdala subnuclei in learned autonomic and behavioural responses to stress (Baklavadzhyan et al., 2000; Iwata et al., 1987; Kapp et al., 1979), some have proposed that rodent IL inhibits stress-related cardiovascular responses by down-regulating the amygdala (Myers-Schulz and Koenigs, 2012).

1.3.2.2 Evidence from NHPs

Two main studies have investigated the contribution of NHP vmPFC to Pavlovian appetitive and negative arousal using different autonomic measures. Rudebeck and colleagues used an appetitive Pavlovian conditioning procedure to investigate the consequences of aspiration lesions of sgACC/25 in macaques on autonomic arousal (pupil size) during CS and US periods (Rudebeck et al., 2014). Whilst lesioned monkeys still showed CS+ evoked autonomic arousal as indexed by increased pupil size, during a trace interval the lesioned animals could not sustain this arousal. As mentioned previously, this study employed ablative lesions and therefore the effects may be due to damage to fibres of passage in the white matter underlying sgACC/25. [Work described next was published in *Proceedings of the National Academy of Sciences, USA* in 2017 with the present author as a co-author: (Wallis et al., 2017)] Wallis and colleagues used targeted pharmacological manipulations of marmoset vmPFC to reveal differential contributions of sgACC/25 and pgACC/32 to stress-evoked cardiovascular arousal during a discriminative aversive Pavlovian conditioning procedure (Wallis et al., 2017). Whilst inactivations of sgACC/25 impaired fear conditioning as evidenced by a reduction in anticipatory cardiovascular arousal to a CS+ predicting an aversive US, pgACC/32 inactivation impaired fear conditioning as evidenced by a generalisation of cardiovascular responding previously reserved to the CS+, to a CS-. These manipulations were without effect on cardiovascular arousal associated with the US. These results illustrate that subregions of primate vmPFC are not only involved in baseline cardiovascular regulation but also in the dynamic adjustment of cardiovascular output during situations of emotional valence – specifically, during appetitive and aversive Pavlovian conditioning. The effects of targeted over-activations within marmoset vmPFC on cardiovascular arousal during appetitive and aversive conditioning are detailed in **Chapter 4** and **Chapter 5** of this thesis, respectively.

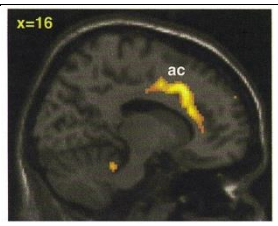
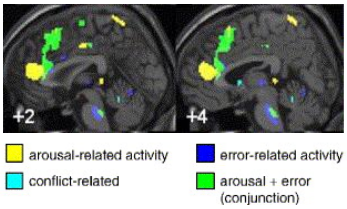
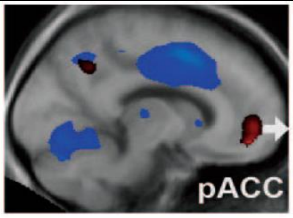
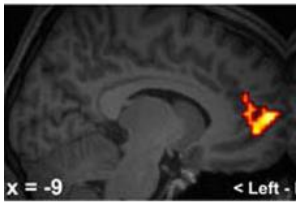
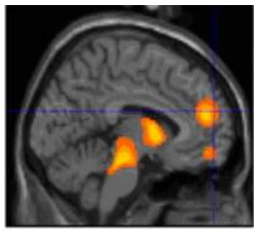
1.3.2.3 Evidence from humans

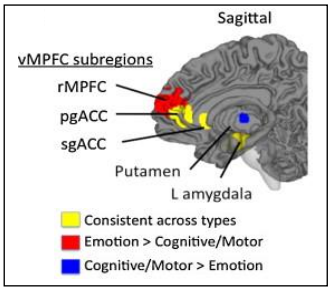
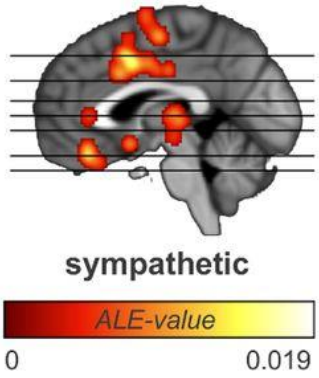
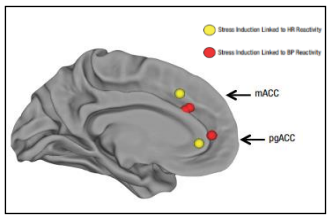
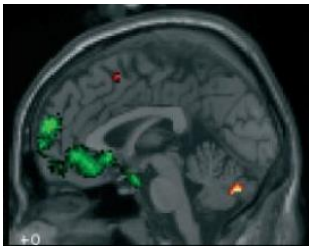
The importance of cortical modulatory influences on autonomic and cardiovascular function during emotion was first highlighted by Albrecht von Haller in 1786, in his seminal work *First Lines of Physiology*:

> *"…terror from a present evil increases the strength of the force of the heart to so great a degree, as to cause convulsions and a strong pulse; whence it [can] kill suddenly."* (von Haller, 1786)

Nineteenth-century physiologists including Walter Cannon and Claude Bernard also documented case reports of 'voodoo death' where a profound emotional state triggered a sympathetic *"emergency reaction*" inducing a *"disastrous fall of blood pressure, ending in death"* (Cannon, 1942).

The advent of neuroimaging meant that specific regions of the brain could be implicated in cardiovascular changes associated with emotional experience and emotional stress. The human mPFC has been implicated in emotion-related changes in cardiovascular activity – the areas typically implicated include both ventral (vmPFC) and dorsal (dACC/dmPFC) regions (**TABLE 1-4**). Indeed, several recent meta-analyses have implicated vmPFC and dACC/dmPFC in emotional and cognitive stressor tasks, where changes in activity within these regions is associated with changes in skin conductance, HR, BP and/or HRV (Beissner et al., 2013; Gianaros and Wager, 2015; Thayer et al., 2012).

Reference	Image	Description
dACC/vmPFC *activations* associated with cardiovascular arousal changes during emotional/stressful situations		
(Critchley et al., 2000)	 BA23, 24, 32	rCBF, BP: activity in dACC (BA23/24) extending into pgACC/32 covaried with BP changes during a mental arithmetic task (and during volitional exercise).
(Critchley et al., 2005)	 BA24, 32, 8, 9	fMRI, pupil diameter: activity within a region spanning dmPFC/dACC predicts Stroop task trial-by-trial variation in autonomic response magnitudes and is enhanced after errors. Activity within pgACC/32 predicted evoked autonomic arousal, independent of errors.
(Gianaros et al., 2007)	 BA 10, 32	fMRI, BP: During a Stroop task, individuals showing higher BP arousal show increased activity in pgACC, in addition to the posterior cingulate cortex and insula.
(Gianaros et al., 2008)	 BA10, 32	fMRI, BP: region of pgACC showing greater BOLD activation (together with lower grey matter volume) associated with increased BP reactivity during a Stroop task. This region also shows elevated functional connectivity with the left and right amygdalae.
(Lane et al., 2009)	 BA10, 32	rCBF, HRV: A region of rostral dACC/ vmPFC (BA10) showing correlated increases in rCBF associated with emotional experience and the high frequency (parasympathetic) component of HRV.

(Thayer et al., 2012)	 BA24, 25, 32	Meta-analysis, rCBF and HRV: changes in rCBF in vmPFC – specifically, right pgACC/24, right pgACC/32 and right sgACC/25 – shows significant associations with changes in HRV across different elicitation methods (both emotional and cognitive/motor).
(Beissner et al., 2013)	 BA10, 24, 25, 32 (all autonomic) BA14, 32 (sympathetic)	Meta-analysis, GSR and HRV: Activation likelihood meta-analysis showing pgACC/32 and sgACC/10, 25 activation in a 'pooled analysis' of all studies showing brain region activation involved in autonomic processing. pgACC/32 and BA14 activation was associated specifically with sympathetic regulation (as measured by GSR), and during tasks associated with cognitive stress.
(Gianaros and Wager, 2015)	 BA24, 32	Meta-analysis, HR and BP: Regions of vmPFC (pgACC) and dmPFC/dACC show activity changes associated both HR and BP responses to social and cognitive stressors.
<u>dACC/vmPFC *deactivations* (or lesions) associated with cardiovascular arousal changes during emotional/stressful situations</u>		
(Gianaros et al., 2004)	 BA 10, 14, 24, 25, 32	rCBF, HR and HRV: increasing difficulty of working memory tasks increased HR and decreased high frequency (parasympathetic) component of HRV – these changes were associated with decreases in rCBF to vmPFC subregions including sgACC/14, sgACC/25 and pgACC/32.

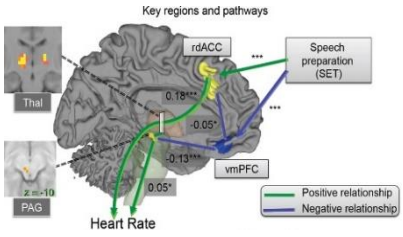
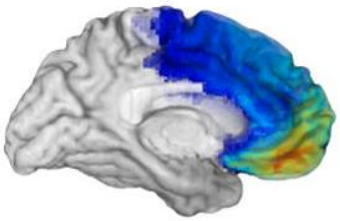
(Wager et al., 2009)	 BA10, 14	fMRI, HR: brain activation and cardiovascular arousal measured during a social threat test (speech preparation). dACC/dmPFC region shows increased activity associated with increased HR during social threat, whereas vmPFC region shows decreased activity associated with increased HR.
(Buchanan et al., 2010)	 BA10, 14, 25, 32	Lesions, HR and HRV: [NB/ Response to stress and heart rate responses assessed independently] Damage to vmPFC causes higher resting HR in men and results in higher self-reports of stress in a social stress challenge.

Table 1-4 Studies implicating human vmPFC in cardiovascular modulation during emotional/stressful situations. In general, these studies show *activation* of a dorsal region corresponding to pgACC/dACC/dmPFC associated with cardiovascular regulation, but *deactivation* of a ventral region corresponding to BA10, 14 and sgACC/25. However, the intensity and direction of change seems to depend on the elicitation method.

Whilst several studies show dorsal *activations* and ventral *deactivations* associated with autonomic outputs during stress (**TABLE 1-4**), the direction and extent of activity change appears to depend on the index of autonomic activity (skin conductance, HR, BP) and the elicitation method (cognitive stress, emotional stress *etc.*; for example, see (Shoemaker and Goswami, 2015)). Beyond a recognition that ventral and dorsal prefrontal regions are important in the interaction between cardiovascular activity, behaviour and emotion (Buchanan et al., 2010; Hilz et al., 2006), it is difficult to ascribe a precise function to specific subregions. Nevertheless, complementary functions of dorsal and ventral aspects of the cingulate gyrus have been suggested based on existing evidence (Bush et al., 2000): the dACC/dmPFC appears to be involved in energy expenditure and regulating sympathetic outflow (Critchley et al., 2003) whereas subgenual regions are involved in parasympathetic tone, behavioural withdrawal, energy conservation and the promotion of safety behaviours (Matthews et al., 2005; Yang et al., 2009).

Several studies in humans have focused on the cortical correlates of changes in HRV. Determining the neural correlates of HRV fluctuations is particularly useful because (i) final

HRV is determined by both parasympathetic and sympathetic contributions which can be fractionated (e.g. (Toichi et al., 1997)); (ii) HRV is linked to trait differences in emotional regulation (Thayer et al., 2012); and (iii) HRV is linked to successful performance in tasks requiring emotional regulation (Park and Thayer, 2014). Thayer and Lane have proposed the *Neurovisceral Integration Model* as a framework to understand how affective and HRV-indexed autonomic flexibility are linked (**FIGURE 1-17**) (Thayer and Lane, 2000). This model builds upon the *Polyvagal Theory* proposed by Porges which focuses on the key role of vagal outputs from the nucleus ambiguus/dorsal motor nucleus in social and affective behaviours (Porges, 1995). In NVI, a core set of neural structures upstream from brainstem vagal motor nuclei – vmPFC and insula – are important in integrating internal and external signals to dynamically adjust physiological parameters by modulating the vagus. By modulating vagal tone, these structures exert additional, critical influences over cognition, perception and action beyond the direct impact of their cortico-cortical connections. The importance of vagal tone is evident both physiologically and cognitively/behaviourally:

- Physiologically, the vagus nerve has negative chronotropic and dromotropic influences on the heart (slowing conduction at the sinoatrial node and atrioventricular node respectively) which are associated with visceral flexibility and sensitivity (holding physiological systems within a sensitive portion of their dynamic range) (Levy, 1990; Porges, 1992).
- Vagal tone can be considered a psychophysiological resource that organisms can utilise in response to environmental challenge – it can be increased or decreased, allowing for the interruption of ongoing behaviours (both during parasympathetic withdrawal and during elevated parasympathetic tone) and re-deployment of attentional and cognitive resources to other tasks.

Caudal regions of vmPFC – particularly sgACC/25 – have been implicated in flexible adjustments in vagal tone associated with emotion regulation. Lane and colleagues have demonstrated strong positive correlations between changes in vagal tone and fMRI BOLD signals in right sgACC/25 during affective state-shifting (Lane et al., 2013).

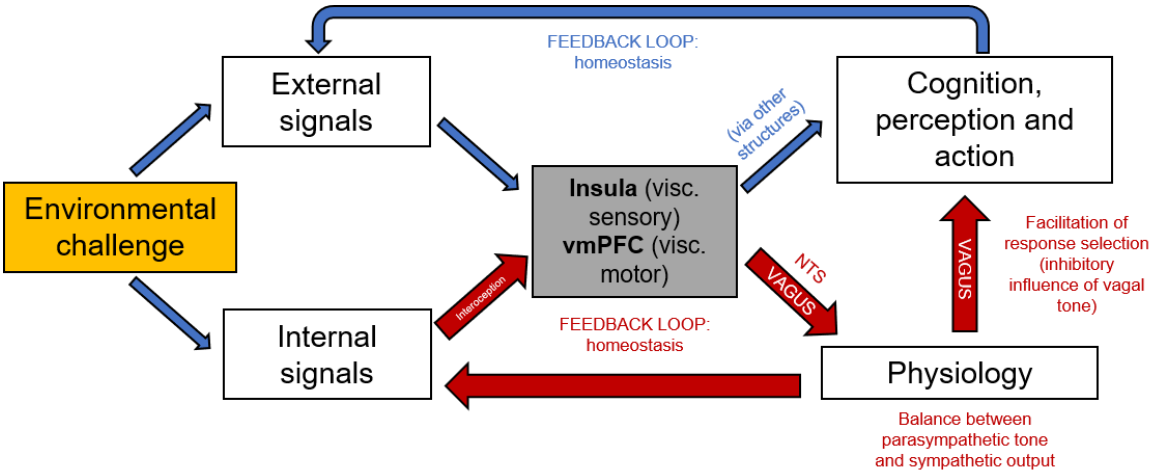


Figure 1-17 Neurovisceral integration (NVI) model. The NVI model of Thayer and Lane (2000) is a framework to understand how the flexible and dynamic adjustment of cognition, emotion, behaviour and autonomic function is linked. Two key cortical structures are suggested as having a key role: the insula and the vmPFC. Environmental challenges produce changes in the external and internal environments which an organism can detect. The insula integrates sensory cues from the internal environment – the interoceptive function of the insula has led to it being labelled 'visceral sensory cortex' – and external sensory cues. Outputs from insula cortex feed into the vmPFC – 'visceral motor cortex' – which can adjust physiological parameters by altering vagal tone (through efferent connections to NTS and the dorsal motor nucleus of the vagus). Critically, feedback relayed via the vagus facilitates flexible response selection during conditions of change as it has an inhibitory influence over behaviour – 'vagal tone' is a psychophysiological resource that organisms can utilise when an ongoing behaviour needs to be stopped and re-adjusted. Changes in peripheral physiology again manifest as interoceptive signals sensed by the insula, which can then readjust parasympathetic tone in the context of external signals forming a negative feedback loop to maintain homeostasis. Changes in cognition and action can modulate external signals, which are then also re-evaluated by central mechanisms. Thus, internal and external signals can be recognised and reacted to in cognitive, behavioural and physiological domains.

1.3.3 Ventromedial prefrontal cortex regulates stress responses and HPA axis activity

Beyond modulation of the autonomic nervous system (ANS), the vmPFC appears to play a critical role in the modulation of another physiological function: the hypothalamo-pituitary-adrenal (HPA) axis (**FIGURE 1-18**). Corticotropin releasing hormone (CRH)-containing neurons of the hypothalamic paraventricular nucleus (PVN) receive inputs from the limbic system, including the amygdala, bed nucleus of the stria terminalis (BNST) and vmPFC.

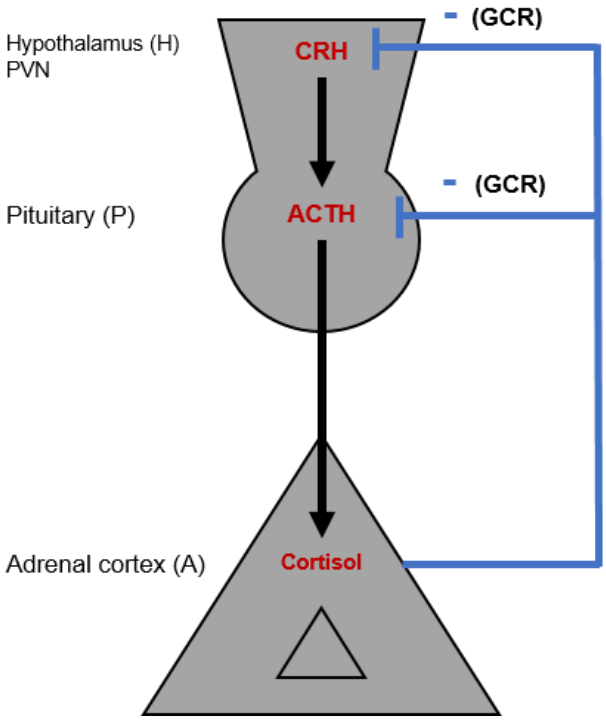


Figure 1-18 The hypothalamo-pituitary adrenal (HPA) axis. Neurons of the paraventricular nucleus (PVN) of the hypothalamus release corticotropin releasing hormone (CRH) into the portal system of the anterior pituitary. These act on secretory cells to release adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH acts on cells of the zona glomerulosa of the adrenal cortex to release glucocorticoids – the most important of which is cortisol. Cortisol is a steroid hormone and permeates all cell membranes to have profound peripheral and central effects. Cortisol forms part of a negative feedback loop to regulate the production of CRH and ACTH, mediated by the glucocorticoid receptor (GCR). This negative feedback loop can be exploited clinically using the dexamethasone suppression test to assess adrenal cortical function. Exogenous administration of dexamethasone (a glucocorticoid) should suppress cortisol production via negative feedback. Failure of suppression indicates insensitivity of the negative feedback elements of the axis as part of e.g. Cushing's disease. Historically the test has also been used to diagnose depression.

1.3.3.1 Evidence from rodents

Following identification of a high density of glucocorticoid receptors (GCR) in the vmPFC of the rat (McEwen et al., 1986; Meaney et al., 1985), work in the 1990s began to reveal the functional contributions of rodent vmPFC to the regulation of HPA function during basal and stressful conditions. Radiofrequency ablation of caudal IL *increases* adrenocorticotropic hormone (ACTH)/cortisol levels after restraint stress but has no effect on baseline cortisol levels, and cortisol implants into IL *reduce* ACTH/cortisol levels after restraint stress with no effect on baseline levels (Diorio et al., 1993). Excitotoxic lesions affecting both PL and IL reduce peak cortisol responses associated with repeat stress, again with minimal effects on baseline cortisol levels (Sullivan and Gratton, 1999).

These early studies, together with work mentioned previously (**1.2.3.1.3**) concerning the role of vmPFC in stress controllability (Amat et al., 2005), have led to an appreciation of the importance of rodent vmPFC in stress regulation. Herman and colleagues have proposed four key characteristics of limbic (including vmPFC, amygdala and hippocampus) regulation of the HPA axis in the rodent (Herman et al., 2005; Jankord and Herman, 2008):

- **The role of limbic structures is region-** (e.g. PL *vs.* IL) **and stimulus-specific** (specific *types* of stress). All sectors of rodent vmPFC show robust cFos induction (an immediate early gene marker of neuronal activity) and enhanced glucose uptake following acute exposure to numerous stressors (Jankord and Herman, 2008). Lesions of PL enhance stress responsivity and stress-associated activation of PVN neurons, whereas lesions of IL attenuate these responses (Radley et al., 2006). Based on studies such as these, IL has been implicated in stimulating stress responses whereas PL has been suggested to inhibit stress responses (Ulrich-Lai and Herman, 2009; Veer et al., 2012). However, the response of IL in particular is stimulus-specific: whilst lesions of IL enhance ACTH responses and PVN cFos activation following administration of the inflammatory cytokine interleukin-1β, they *reduce* restraint-induced PVN cFos activation (Crane et al., 2003). Evidence also suggests lateralisation of function within the vmPFC, with right vmPFC being most directly linked to HPA axis regulation (Cerqueira et al., 2008; Sullivan and Gratton, 1999, 2002).
- **There are minimal direct projections from limbic structures to PVN CRH neurons – projections relay in the BNST, NTS or dorsomedial nucleus of the hypothalamus (DMH).** PL sends glutamatergic projections to the aBNST, which inhibits PVN and reduces the cortisol response to acute stress. IL projects directly to aBNST, DMH and NTS, which input to PVN. The targets of IL are differentially activated by psychogenic *vs.* systemic stressors (Cullinan et al., 1995; Emmert and Herman, 1999; Figueiredo et al., 2003; Sawchenko et al., 2000; Thrivikraman et al., 2000). Furthermore, evidence suggest that in some of these structures – particularly the NTS – different cell populations are activated by psychogenic *vs.* systemic stressors. This provides evidence downstream of IL showing that neural structures critical in autonomic control show modality-specific responses (Dayas et al., 2001; Jankord and Herman, 2008).
- **There is extensive overlap between vmPFC, amygdala and hippocampal projections onto the BNST suggesting there is integration at subcortical relay sites**. Regulation of the HPA axis is a distributed process involving the hippocampus, amygdala and IL/PL. Evidence suggests that the BNST serves as an integration structure, receiving converging inputs from this diverse array of structures and sending efferents to the PVN to regulate HPA axis output (Jankord and Herman, 2008).
- **Limbic structures have divergent projections to multiple subcortical targets** (BNST, DMH).

Precise determination of the role of vmPFC subregions in HPA axis regulation is complicated by the fact that these regions also participate in negative feedback loops controlling cortisol levels: for instance, glucocorticoids appear to act on PL to regulate negative feedback in acute situations of stress only, glucocorticoids act on IL to regulate negative feedback during *both* acute and chronic stress (McKlveen et al., 2013). Therefore, glucocorticoids act via both PL and IL during acute negative feedback, whereas feedback during chronic stress is mediated solely by IL. These signalling pathways impact upon affective behaviour, as GCR knockdown in IL (but not PL) increases immobility time in the forced-swim test, a common assay of depression-like behaviour.

1.3.3.2 Evidence from NHPs

Immunohistochemical assessment of GCR/mineralocorticoid receptor (MCR) expression in the cortices of NHP species (such as the squirrel monkey and rhesus macaque) have revealed high densities of immunoreactive nuclei distributed throughout the prefrontal cortex, particularly in ventromedial and lateral subregions (Patel et al., 2000; Sánchez et al., 2000). Despite this, the functional relevance of NHP vmPFC to HPA axis regulation has scarcely been explored. Jahn and colleagues recently examined the brain circuitry involved in individual differences in HPA axis regulation in macaques – specifically, the relationship between regional cerebral metabolic activity and cortisol levels across contexts with differing affective valence (Jahn et al., 2010). Macaques were exposed to four situations of increasing stress: home with cage-mate, home alone, human intruder exposure or foreign cage alone. After 30 minutes in these situations, macaques underwent femoral venepuncture for cortisol levels and an ^{18}F-FDG PET scan. Using logical and conjunctional analyses, Jahn and colleagues were able to identify regions where metabolism and cortisol correlated across all conditions. In their analysis, macaque sgACC/25 was the only brain region showing activity correlated with cortisol output across different contexts. It is difficult to infer precise meaning from this correlation, because the positive relationship likely reflects a combination of stimulatory and inhibitory (negative feedback) processes co-occurring within the HPA axis. For instance, sgACC/25 activity could be correlated with cortisol output if (i) it were providing a direct stimulatory input to the HPA axis, or (ii) sgACC/25 was activated by increasing concentrations of circulating cortisol to exert negative feedback and supress further responses.

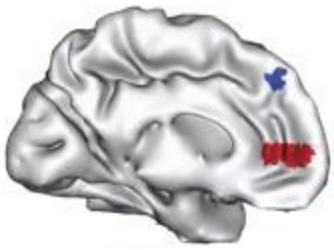
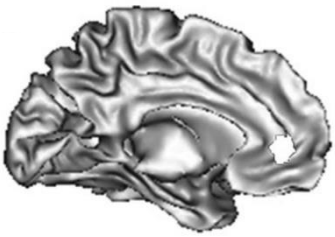
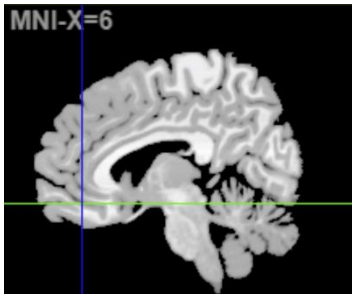
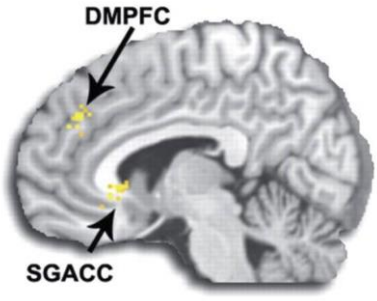
1.3.3.3 Evidence from humans

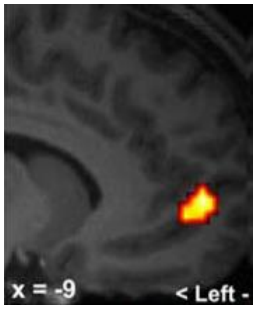
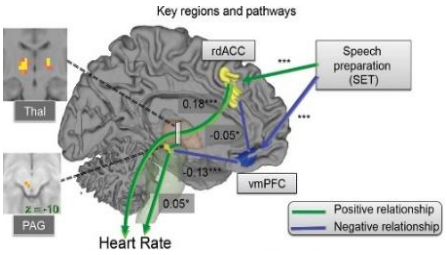
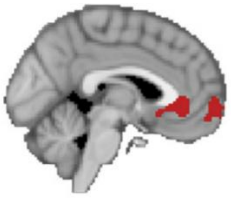
Surprisingly few studies have examined the neural correlates of HPA axis regulation in humans (see **TABLE 1-5** for examples). Of the studies conducted, most focus on vmPFC-amygdala connectivity given (i) the key role of the amygdala in facilitating stress responses

and (ii) that projections from vmPFC to amygdala are involved in inhibitory feedback and top-down regulation of emotion and stress (Herman et al., 2005).

These studies consistently implicate functional connectivity between a perigenual region of the vmPFC – typically encompassing pgACC/32, pgACC/24 and sometimes extending to sgACC – and the amygdala in responses to stressful situations and emotional conflict. These activations are interpreted in the context of top-down regulation of amygdala activity, as activation is seen when comparing effortful affective regulation versus baseline conditions (Banks et al., 2007; Urry et al., 2006; Veer et al., 2012). Activity within overlapping vmPFC regions has also been associated with fluctuations in daily cortisol levels (Hakamata et al., 2017; Veer et al., 2012), and exogeneous cortisol administration has been shown to directly modulate sgACC/25 activity (Sudheimer et al., 2013) implying that there is a bidirectional relationship between prefrontal structures and peripheral correlates of HPA axis activity.

A small number of studies have also shown differential patterns of human vmPFC activation associated with different types of stress. Ohira and colleagues identified a region of pgACC corresponding to pgACC/32 in which rCBF was directly related to the redistribution of natural killer cells (an immunological reaction to stress) during situations of controllable stress (monetary gain *vs.* loss) (Ohira et al., 2009). Activity in a caudal region of sgACC corresponding to sgACC/25 (and caudal BA10) is associated with increased interleukin-1β concentrations during presentation of grief related words (O'Connor et al., 2009). Similarly, Harrison and colleagues have shown that increases in interleukin-6 levels following typhoid vaccination are associated with mood-reductions, and these negative changes in mood are directly associated with enhanced sgACC/25 activity (Harrison et al., 2009). Although further studies are needed, the data from these select few imply activity in a subgenual region related to inflammation-induced stress, and activity in a perigenual region associated with stress related to cognitive challenge.

Reference	Image	Description
	Human vmPFC in stress regulation	
(Etkin et al., 2006)	 Emotional conflict: BA8,9 Resolution: BA32	fMRI, emotional conflict: Activity in dmPFC (BA8/9) associated with emotional conflict, whereas activation of pgACC/32 (and BA10) is associated with the resolution of emotional conflict. PgACC/32 activation is accompanied by a correlated reduction in amygdala activity, suggesting top-down regulation of the amygdala by pgACC/32.
(Egner et al., 2008)	 Resolution: BA32	fMRI, emotional conflict: pgACC/32 part of an 'emotional control' system – activation associated with the resolution of emotional conflict and decreased amygdala responses.
(Urry et al., 2006)	 BA11	[Location of peak activation] fMRI, negative picture stimuli: individuals showing reduced amygdala responses when instructed to decrease their affective responses to negative picture stimuli also show higher signal in the vmPFC. These individuals also show steeper declines in cortisol levels over the course of the day.
(Banks et al., 2007)	 BA25	fMRI, negative picture stimuli: activity within sgACC/25 and dmPFC/8,9 covaries with amygdala activity during active reappraisal of negative picture stimuli. The strength of connectivity predicts the extent of attenuation of negative affect after reappraisal.

(Gianaros et al., 2008)	 BA10, 32	fMRI, stressor-evoked BP: area of pgACC/32 and BA10 showing higher connectivity with the right amygdala associated with greater BP reactivity during a Stroop stressor task.
(Wager et al., 2009)	 BA14, 25	fMRI, stressor-evoked HR: brain activation and cardiovascular arousal measured during a social threat test (speech preparation). dACC/dmPFC region shows increased activity associated with increased HR during social threat, whereas vmPFC region shows decreased activity associated with increased HR. Also see section on cardiovascular regulation.
(Veer et al., 2012)	 BA25, 32, 10	fMRI, cortisol levels: Stronger negative functional connectivity between sgACC/25 and pgACC/32 and the amygdala is associated with higher cortisol levels. These regions are likely to be involved in the top down regulation of the amygdala during the stress response.
(Sudheimer et al., 2013)	 BA14, 25	fMRI, negative picture stimuli: single dose and extended dose of cortisol (administered peripherally) block the increase in sgACC/25 activity evoked by sadness (placebo groups show an increase in sgACC/25 activity, whereas groups given cortisol do not).

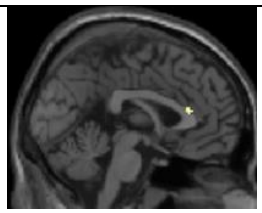
(Hakamata et al., 2017)	 BA24	fMRI, fearful faces: functional connectivity between 'pgACC' (dACC/24) and amygdala negatively correlated with daily cortisol levels, and negatively correlated when processing fearful faces.

Table 1-5 Studies implicating human vmPFC in the regulation of the stress response. Several of these studies show pgACC/32 and sgACC/25 activity covaries with amygdala activity during situations of stress: specifically, situations of emotional reappraisal typically involve upregulated vmPFC activity and downregulated amygdala activity. These functional imaging changes are associated with peripheral cardiovascular and endocrine (cortisol) sequalae.

1.4 Specialisation of Function Within the Ventromedial Prefrontal Cortex?

The thesis so far has discussed the role of the vmPFC in reward processing and value-based decision-making; in the regulation of negative emotion (including pain); in social cognition; in cardiovascular regulation; and in regulation of the HPA axis. The vmPFC is, therefore, implicated in functions across cognitive, behavioural and physiological domains. Two hypotheses have emerged attempting to explain the myriad of functions the vmPFC is involved in:

- Given the anatomical heterogeneity within the vmPFC, together with heterogeneity in implicated function, it would be reasonable to suggest that anatomical subregions within the vmPFC have associated functional specialisation (Hiser and Koenigs, 2018).
- Whilst the vmPFC is anatomically heterogeneous, the subregions are extensively connected with one another (Price and Drevets, 2010). Intra-vmPFC connectivity is critical in integrating a diverse array of inputs, such that the vmPFC acts as a *hub* of interrelated systems involved in memory, social cognition, interoception and autonomic regulation (Roy et al., 2012)

First, a consideration of hypothesis one. In their review of vmPFC function, Hiser and Koenigs downloaded a series of meta-analyses using *NeuroSynth* (www.neurosynth.org) (Yarkoni et al., 2011), each using search terms associated with a particular function – value/decision-making, emotion or social cognition. Using this strategy, they could identify subregions of the vmPFC, together with other structures, whose activity is related to these functions across many studies. Their analysis is shown in **Figure 1-19A**. Value and decision-making was associated with activity in anterior BA10, sgACC/25 and the ventral striatum; emotion was associated with sgACC/25 and caudal BA10 together with the amygdala; and social cognition with anterior BA10 and dmPFC, precuneus and temporo-parietal cortex. These data suggest distinct clusters of activity within vmPFC associated with distinct functions, although there is some overlap – particularly within sgACC/25. Whilst informative, one must also be mindful of the caveats associated with this approach, including the choice of search terms. For example, in their assessment of clusters involved in value/decision-making, Hiser and Koenigs used the terms ‘value,’ ‘reward’ and ‘decision-making.’ Firstly, these constructs are not necessarily part of one homogenous function, and second, these search terms omit key constructs related of reward processing such as reward anticipation and reward motivation.

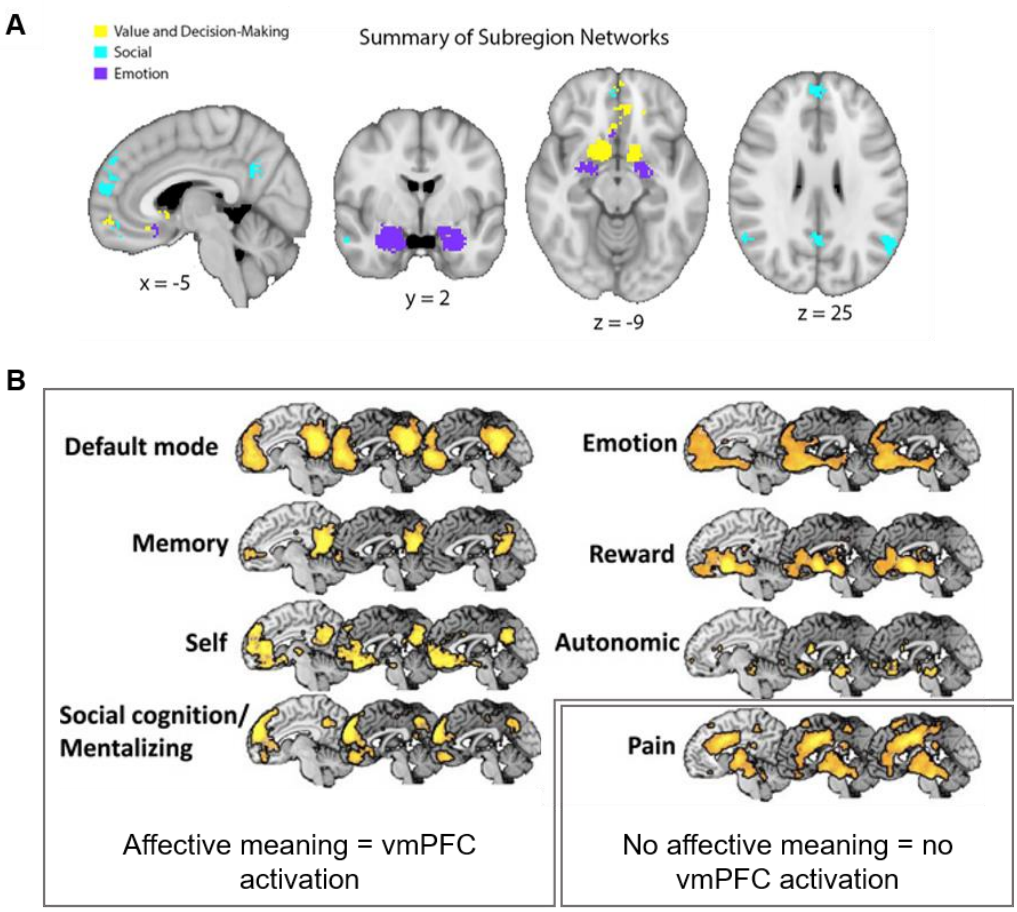


Figure 1-19 Perspectives on vmPFC function. **A** Adapted from Hiser and Koenigs (2018). Based on their meta-analyses carried out using search terms with the *Neurosynth* software, Hiser and Koenigs propose that distinct subregions within the vmPFC are involved in value/decision-making *vs.* social processing *vs.* emotion. These vmPFC subregions are also associated with distinct patterns of co-activation with other structures. **B** Adapted from Roy *et al.* (2012). Roy and colleagues suggest that the vmPFC is responsible for extracting 'affective meaning' from a diverse constellation of external and internal stimuli to create a unitary representation termed a 'schema', and co-ordinating the appropriate behavioural and physiological response. When analogous physiological and behavioural outputs are induced but the triggering stimulus is unconditionally aversive, and its valence does not require integration of internal/external cues ('conceptual' information) – such as pain – the vmPFC is not activated.

Next, consider hypothesis two. Roy *et al.* suggest that the vmPFC is a 'hub' region which links cognitive and affective information with physiological and behavioural responses. By integrating a constellation of external and internal cues, the vmPFC can extract 'meaning' from a situation and transduce this meaning into outputs critical for an organism's survival. To construct this meaning, Roy and colleagues suggest the vmPFC must be involved in several processes: constructing unitary representations of a situation ('schema') from configurations of cues; recalling past situations and abstracting features to guide prediction

about future outcomes; evaluating the potential outcomes in terms of benefit/harm to the 'self'; and triggering appropriate physiological and behavioural responses (or modifying ongoing ones). A central tenet of their hypothesis, therefore, is that vmPFC is essential when *conceptual* information (external cues and self-relevant information – schema) is driving affective physiological/behavioural processes. Using a similar *NeuroSynth*-based meta-analytic approach, Roy *et al*. extracted maps of brain networks whose activity was specifically associated with functional tasks related to affective meaning. Studies included brain changes related to memory, self-referential function (DMN), social cognition, emotion, reward and physiological (autonomic/endocrine) changes. By contrast, exposure to painful stimuli induces behavioural/physiological changes without a need for representation of affective meaning (it is unconditionally aversive). Shown in **FIGURE 1-19B**, Roy and colleagues found that there was overlapping vmPFC activation across all 'meaning-related' domains, but the pain map was largely distinct from the others, primarily involving dACC. They propose that this overlapping activation represents a common function of vmPFC in affective appraisal, across situations with different valence.

Although there is extensive overlap, Roy *et al*. do acknowledge that there is some degree of functional specialisation in different parts of the vmPFC. For instance, DMN and memory maps include PCC whereas the emotion, reward and autonomic maps include the ventral striatum and amygdala. Inspection of the maps in **FIGURE 1-19B** suggests that there are at least two distinct subsystems overlapping on the vmPFC. The first subsystem includes anterodorsal vmPFC, dACC, dmPFC and PCC and is highlighted in the DMN and memory maps. This subsystem seems to be involved in internalisation, self-referential processes and constructing internal models of the world to imagine projected future scenes (Buckner et al., 2008). The second subsystem includes ventral, caudal vmPFC (including sgACC/25) and is highlighted in the reward, autonomic and emotion maps. Understanding these subsystems, their constituent zones and their differential roles – whether they are truly distinct, or ultimately subserve one emergent function – is an ongoing quest. Specialisation could be related to content (positive *vs.* negative emotion), process (response selection *vs.* value updating), output (skeletomotor, autonomic, endocrine *etc.*) or another dimension entirely. Furthermore, the relationship of these functional zones to cytoarchitectonic, myeloarchitectonic and chemoarchitectonic properties is still not clear (nor indeed, whether there is any relationship at all).

The role of the vmPFC to generate 'schema' as suggested by Roy and colleagues is closely related to the proposed function of vmPFC as proposed by Antonio Damasio and Antoine Bechara in the somatic marker hypothesis, discussed previously. They propose that the vmPFC is:

"… a repository of dispositionally recorded linkages between factual knowledge and bioregulatory states." (Bechara et al., 2000)

In their proposal, the vmPFC and its constituent subregions are responsible for forming associations between situations (consisting of actors and actions, response options and consequences of each response option) and the subjective and physiological (emotional) states associated with those situations based on past experience. The different components of the situation are 'dispositionally linked' to an emotional response, and the vmPFC is the key site for the link between specific components and their emotional responses based on previous learning. Their proposal is very similar to that suggested by Roy and colleagues – in particular because it ascribes a common function to the diverse array of subregions comprising the vmPFC.

1.5 Psychiatric Disorders and the Ventromedial Prefrontal Cortex

Given the role of the vmPFC in emotion and its regulation, it is not surprising that dysfunction within the vmPFC has been implicated in the aetiology and pathogenesis of psychiatric disorders. Indeed, meta-analyses of fMRI brain activation across several different psychiatric disorders consistently implicate distinct but overlapping subregions of the vmPFC (Mayberg et al., 1999). This thesis shall focus its discussion on major depressive disorder (MDD) and anxiety disorders.

1.5.1 Depression and Major Depressive Disorder

If a fright or despondency lasts for a long time,

it is a melancholic affection.

Hippocrates, Aphorisms, Section 6, no. 23

1.5.1.1 Defining Depression

Depression is not a unitary construct and it is perhaps more appropriate to think of a range of *depressive disorders* with differing severity. These disorders are characterised by a state of persistent low mood, loss of interest/enjoyment, neuro-vegetative disturbances and reduced energy, resulting in varying levels of occupational and social dysfunction.

1.5.1.2 Classifying Depression

The DSM-V divides depressive disorders into four subtypes (American Psychiatric Association, 2013): MDD (also termed unipolar depression); persistent depressive disorder (dysthymia); premenstrual dysphoric disorder; and 'other' depressive disorders (due to side effects of drugs, substance abuse, medical conditions or other unspecified causes). Depressive symptoms are also seen as part of cyclothymia or bipolar disorder, where there are alternating manic/depressive phases. Besides a difference in cause, the clinical category of depression is also distinguished by the length of symptoms, the number of auxiliary symptoms (beyond core symptoms of low mood and/or anhedonia), the degree of functional impairment and the symptom severity. Nevertheless, it is becoming increasingly appreciated that there may be similarities in the pathophysiological changes at the heart of these disorders.

MDD is the archetypal mood disorder, involving a persistent (longer than two weeks) change in mood, cognition and physiological functions. The Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) criteria are used in both research and clinical settings

to stratify depression severity. The criteria for MDD are shown in **FIGURE 1-20**. Criterion symptoms (highlighted, **FIGURE 1-20**) must be present nearly every day for most of the day to be considered as present. MDD episodes are discrete but may last a long time (if the mood disturbance continues for longer than two years, MDD becomes persistent depressive disorder) with remissions between episodes. In this thesis, the term 'depression' will be used synonymously with MDD, although again it is important to emphasise that there are likely commonalities in the neural changes associated with the different subtypes of depression outlined by the DSM-V.

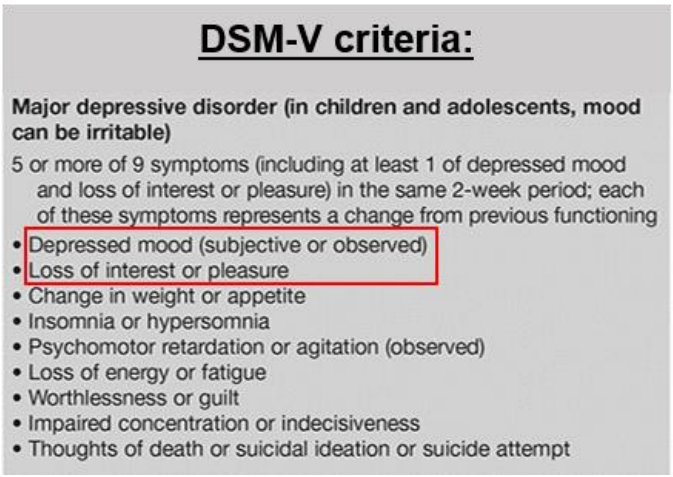


Figure 1-20 DSM-V criteria for diagnosing MDD. American Psychiatric Association, 2013. Criterion symptoms are highlighted.

1.5.1.3 Epidemiology of Depression

Depressive disorders are extremely common, and have been recognised as a leading cause of disability worldwide (Ferrari et al., 2013; Kessler et al., 2009; Murray and Lopez, 1997; Qaseem et al., 2016). Depression is predicted to be the second leading cause of disability in people of all ages by 2020 and current estimates suggest 20% of adults will be affected by a mood disorder at some point during their life with MDD accounting for half of these diagnoses (Remick, 2002). The age of onset is bimodal, with incidence peaks typically between 12 and 24 years or in those older than 64 years (Remick, 2002). Women are affected twice as often as men (Albert, 2015).

1.5.1.4 Aetiology and Pathophysiology of Depression

The aetiology of depression is complex and is still poorly understood. It affects people of all ages, varies in its duration and severity, and differentially affects men and women. This heterogeneity would suggest that the aetiology of depression involves multiple factors. Neurobiologically, whilst it is generally accepted that there is some form of neurobiological

change/'biochemical imbalance' involved, it is also appreciated that there is no single brain region or neurotransmitter system which is *always* dysfunctional in depressed patients.

1.5.1.4.1 Behavioural accounts

One of the most influential behavioural accounts of the aetiology of depression was proposed by Martin Seligman in his theory of *learned helplessness* (mentioned previously in the context of stress controllability) (Seligman, 1974). This suggests that depression develops when individuals learn that their attempts to escape negative situations make no difference. This results in passivity: patients will endure aversive stimuli and environments even when escape is possible. This theory evolved following Seligman's behavioural shuttle-box work in dogs (Seligman and Maier, 1967). In phase one of the experiment, two groups of dogs were arranged in yoked pairs – dogs in group A were shocked but could end the shock by pressing a lever, whereas dogs in group B were shocked whenever dogs in group A were shocked, but pedalling did not stop the shock. To group B, the shock seemed to end at random as it was contingent on a dog in group A pressing a lever.

In phase two, dogs were tested in a shuttle-box apparatus where they could escape being shocked on one side of the apparatus by leaping over a small partition into a 'safe zone.' Group A quickly learned the avoidance response. Group B – who had previously experienced uncontrollable shocks – simply sat passively whilst they were shocked, showing lethargy, sluggishness and appetite loss even after the test session. Seligman suggested that the behaviour of dogs in Group B was akin to them having 'given up' trying to change their circumstances. In its explicit formulation, learned helplessness theory is that patients with MDD have a real or perceived absence of control over the outcomes of situations (Seligman, 1975). Subsequent work has shown that the acquisition of learned helplessness is associated with vegetative disturbances – in rats, exposure to uncontrollable shocks results in gastric erosion more readily than the same number of controllable shocks highlighting a link between uncontrolled stress and vegetative disturbances consistent with mood disorders (Murison and Isaksen, 1982).

Learned helplessness has been demonstrated in humans. For instance, Hiroto presented college students with either controllable aversive noise which could be terminated with a button press, or uncontrollable noise whose termination was unrelated to button pressing (Hiroto, 1974). In a second phase, students were tested in a hand-shuttle box where students simply had to move a lever from one side of a box to another to terminate the noise. The results of this study were analogous the study in dogs: students previously receiving prior controllable shocks readily learnt the shuttling response, whereas the group receiving uncontrollable shocks failed to shuttle and listened to the noise passively.

1.5.1.4.2 Cognitive accounts

Cognitive theories of depression focus on the dysfunctional thought patterns which depressed patients adopt. Aaron Beck famously suggested that depression results from a systematic negative bias in thinking, meaning that depressed patients *think differently* to healthy controls and that these changes may even precede the onset of depressed mood (Beck, 1973). A second cognitive account – termed attributional theory – was developed as an extension of Seligman's work on learned helplessness, modified to place more emphasis on the cognitions of a depressed individual (Abramson et al., 1978).

Aaron Beck's account proposes three mechanisms that are involved in the predisposition for and development of depression: negative self-schemas which predispose individuals to developing depression; together with a cognitive triad of negative automatic thinking and errors in logic, both of which constitute the depressed cognitive phenotype.

- **Negative self-schemas** refer to a set of beliefs that are negative and pessimistic. Examples of negative self-schemas include an ineptness schema (expectation to fail), a self-blaming schema (a responsibility for negative events) and a negative self-evaluation schema (feeling worthless). These are acquired in childhood (and carried through to adulthood) following a traumatic event and act to pre-dispose an individual to depression. In a nod to psychosocial theories, Beck proposed that negative life events act to 'activate' these negative schemas, resulting in the cognitive triad and errors in logic. There is evidence for a predisposition to developing depression in individuals who have negative thinking styles. Alloy and colleagues (Alloy et al., 2006) assessed the thinking styles of young Americans for six years, and classified them into a 'positive thinking' group or 'negative thinking' group. The positive thinking group had a 1% rate of depression over the next six years, whereas the negative thinking group had a 17% rate of depression, suggesting that there is a link between thinking styles and the development of depression.
- The **cognitive triad** outlines three forms of negative thinking that typify individuals with depression. These are negative views of the self ('I am worthless,' 'I am inadequate'), negative views of the world (interpreting events in an unrealistically negative and defeatist way) and negative views of the future (situations will not improve). These negative thoughts become all-encompassing and this leads to problems in cognition and perception.
- **Errors in logic** refer to cognitive *biases* that outline dysfunctional changes in attentional deployment associated with the depression – patients are prone to making logical errors by focusing on specific aspects of a situation whilst also ignoring other aspects. Different forms of errors in logic are outlined in **TABLE 1-6**.

Error in logic	Description
Arbitrary inference	Drawing a negative conclusion in the absence of supporting data
Selective abstraction	Focusing on the worst aspects of a situation
Magnification/minimisation	Problems are made bigger and solutions are made smaller
Personalisation	Negative events are interpreted as patient's own fault
Dichotomous thinking	'Black-and-white' thinking

Table 1-6 Examples of errors in logic (cognitive biases) outlined by Aaron Beck's cognitive theory of depression.

A second cognitive account builds on the behavioural mechanisms proposed in learned helplessness theory, which does not consider the cognitions of depressed individuals. To resolve this, learned helplessness theory was reformulated into attributional theory (Abramson et al., 1978) which focuses on how individuals explain the causes of events in their lives – termed attributional styles. Attributional styles have three key components: locus (internal or external), stability (permanent or transient) and specificity (global or specific). Abramson considered the presence of a negative event alone insufficient to produce a depressed or helpless state. Instead, individuals who attribute negative events to internal, stable and global causes are more likely to become depressed. This thinking style leads people to think that they cannot change things for the better. There is ample evidence available to support attributional theory: depressed patients do show internal, stable and global attributional styles (Seligman et al., 1984); these attributional styles change following treatment (Petersen et al., 2004); and cognitive behavioural therapy – an effective treatment for depression – involves tackling these thought patterns (Proudfoot et al., 2004).

A cardinal feature of depressed thinking consistent with both of these cognitive accounts (but not explicitly outlined by either) is rumination: a recurrent, self-reflective focus on depressed mood, its causes and its consequences (Hamilton et al., 2015; Morrow and Nolen-Hoeksema, 1990). High levels of state rumination predict symptom severity (Kuehner and Weber, 1999), and high levels of trait rumination predict symptom onset (Nolen-Hoeksema et al., 2008). Furthermore, the presence of ruminative thinking reliably discriminates between depressed and never-depressed individuals (Hamilton et al., 2011a). Although is becoming increasingly apparent that rumination is a central part of the clinical phenotype of depressed patients (Lyubomirsky et al., 1999), in both the DSM-V and International Classification of Diseases (ICD)-10, it is not considered a criterion symptom of depression. The focus on

ruminative thinking has also seen a resurgence following the recognition of the DMN (see **1.5.3.3**) as a neural network responsible for self-referential processes (Fox et al., 2005). The intuitive link between this function and rumination has led to the development of neural models of rumination which include structures involved in the DMN (Hamilton et al., 2015).

1.5.1.4.3 Genetic accounts

Twin, family and genome-wide association studies have shown that depression is to some extent heritable (Lohoff, 2010).

- **Twin studies**: A meta-analysis of twin studies in 2000 estimated the heritability of depression at 37% (Sullivan et al., 2000).
- **Family studies:** Family studies have shown that first-degree relatives of depressed patients have between a two-fold to four-fold increased risk of developing the disease, relative to controls (Lohoff, 2010; Weissman et al., 1993).
- **Genome-wide association studies (GWAS)**: Single nucleotide polymorphisms contribute to approximately 9% of variation in susceptibility, although the contribution made by polymorphisms in individual genes is very small (Wray et al., 2018). Many of the polymorphisms are related to excitatory neurotransmission and post-synaptic/dendritic function (Howard et al., 2018).

As suggested by the results of GWAS, the genetic contribution to depressive disorders is polygenic, although polymorphisms in serotonin (5HT)-related genes have received the most attention. Deficits in 5HT handling within the central nervous system have been linked to numerous psychiatric disorders, including depression (Neumeister et al., 2004). A functional 44 base-pair repeat polymorphism in the promoter region (5HTTLPR) of the serotonin reuptake transporter (5HTT) has received attention owing to its effect on *in vivo* 5HT levels. Caspi and colleagues have implicated this polymorphism in modulating the influence of stressful life events on depression – specifically, individuals homozygous for the 'short' *s* allele are at increased risk of depression compared to individuals homozygous for the 'long' *l* allele (or *s*/*l* heterozygotes) when exposed to stressful life events (Caspi et al., 2003). This study suggests that a gene × environment interaction contributes to developing depression. Providing a neurobiological instantiation of the differential effect of 5HTT alleles, Hariri and colleagues imaged individuals with polymorphisms of the 5HTTLPR gene and found greater amygdala activation in response to threatening stimuli in patients with the *s* allele (Hariri et al., 2002). This directly implicates the polymorphism in differential sensitivity of the brain's threat processing systems. However, it should be noted that subsequent studies investigating a link between the polymorphism and incidence of depression have yielded equivocal results. Most recently, a large collaborative meta-analysis found no evidence of

any statistically significant interaction between 5HTTLPR allele status and life stressors for the risk of depression (Culverhouse et al., 2017).

The influence of genetic factors is not limited to direct effects on physiology: they also alter the nature of an individual's interaction with the environment (Kendler and Karkowski-Shuman, 1997). Despite the contribution made by genetic factors, the development of MDD involves a strong influence of the environment and tiny contributions of individual genes (Kendler et al., 2006). In the future, genetic testing will provide likely limited information regarding an individual's propensity to develop depression. A deeper understanding of the genetic contributions to developing mood disorders will nonetheless provide us with an insight into the neurobiological and neurochemical mechanisms underlying depression and the potential pathways by which antidepressants act.

1.5.1.4.4 Dysfunction within HPA axis

One major model of the aetiology of depression suggests that dysregulation of the body's response to stress is a key causal mechanism. This includes dysfunctional responses within the HPA axis including abnormal release of CRH and abnormal activation of the noradrenergic (NA) system including the locus coeruleus (LC).

Cortisol is the primary glucocorticoid hormone, triggering an acute stress response which is self-limiting owing to negative feedback loops within the HPA axis. If these negative feedback loops do not function correctly, the resulting high levels of cortisol can have deleterious consequences including enhanced negative mood. Elevated levels of cortisol and CRH have been consistently identified in depressed populations (Plotsky et al., 1998), together with blunted regulation of cortisol following psychological stress (Burke et al., 2005). The associated sustained hypercortisolaemia results in pathological changes to brain structures – particularly the hippocampus (Sapolsky, 2000, 2001) – which has generalised effects on patients' ability to regulate emotion. Further evidence for an association between sustained hypercortisolaemia and depression comes from patients with Cushing's syndrome (caused by excess administration of exogenous steroids or a pituitary tumour [Cushing's disease]), who often exhibit mood disturbances characterised by a depressive-like affective state (Sonino et al., 1998).

Recent work has explored the role of changes to GCR function through epigenetic or inflammatory mechanisms, and the resultant impact this has on HPA axis function. Whilst the mineralocorticoid receptor has a high affinity for cortisol (and therefore operates within its dynamic range at lower cortisol concentrations), the GCR has lower affinity for endogenous cortisol and therefore is thought to be important in stress response regulation when baseline levels of cortisol are higher (as is the case in depression). Converging evidence points to

GCR-related dysfunction in depression (**TABLE 1-7**) which manifests as 'glucocorticoid resistance' exhibited by depressed patients (Anacker et al., 2011). The focus on the GCR is not unwarranted because it is key in mediating the negative feedback loop critical in maintaining normal HPA axis function (see **FIGURE 1-18**). The efficacy of this negative feedback is affected by cortisol availability together with early life trauma, inflammation and (epi)genetic factors which influence GCR function and expression. Changes in the negative feedback loop may be normalised following treatment with antidepressants, and this may (in part) underlie their mechanism of action (Pariante and Lightman, 2008). An increased understanding of how antidepressants modulate responses to glucocorticoids in normal physiology and in the context of depression may lead to a better understanding of components of their efficacious action.

References	GCR-related abnormality
(Bhagwagar et al., 2003, 2005; Juruena et al., 2009; Nemeroff et al., 1992; Pariante, 2009; Young et al., 1991, 2003)	Higher cortisol levels in patients with depression (even after recovery) compared to controls, suggesting changes in feedback loops mediated by GCR: increases in cortisol levels may reflect a compensatory mechanism due to reduced sensitivity of GCRs ('glucocorticoid resistance')
(Hayes and Ettigi, 1983; Heim et al., 2008; Nutt, 2001; The APA Task Force on Laboratory Tests in Psychiatry, 1987)	Failure to suppress cortisol secretion after administration of dexamethasone, suggesting a non-responsive GCR-dependent negative feedback loop (although evidence for this effect is variable)
(Avissar et al., 1997; Bierhaus et al., 2003; Lowy et al., 1984; Maes et al., 1993; Wodarz et al., 1991)	Impaired function of GCR-related pathways in peripheral blood mononuclear cells cultured from depressed patients
(Binder et al., 2004; van Rossum et al., 2006)	Polymorphisms in GCR-related genes predict susceptibility to develop depression together with differential responses to anti-depressants

Table 1-7 Converging lines of evidence point to GCR-related dysfunction in depression. These include higher baseline cortisol levels; failure of dexamethasone suppression; impaired function of GCR pathways in peripheral blood cells; and polymorphisms in GCR-related genes associated with a predisposition to depression and differential treatment response.

Note that changes in HPA activity and reactivity may represent a susceptibility to depression (rather than a cause) that manifests during early life (Pariante and Lightman, 2008). Early maternal separation in rodents and primates can produce long-lasting changes in HPA axis function, namely hyperactivity together with increased activity in CRH-sensitive circuits (Sánchez et al., 2001). A hyperactive HPA axis has also been identified in men with early life trauma using the dexamethasone suppression test (Heim et al., 2008). Epigenetic mechanisms may explain how these early life stressors result in persistent changes in the HPA axis, with current working focusing on cortisol-related genes such as the GCR (van Rossum et al., 2006; Weaver et al., 2006) and CRH (Korosi and Baram, 2008) genes. These

biological mechanisms may explain the correlation between early life stress and adult depression, although epigenetic changes in stress-related genes do not act in isolation and polymorphisms in other genes (e.g. 5HTT, see **1.5.1.4.3**) also interact with negative life events.

1.5.1.4.5 Dysfunction within monoamine systems

The monoamine theory of depression asserts that the underlying pathophysiological basis of depression is depletion in the levels of 5HT, NA and/or dopamine (DA) in the central nervous system. Historically, initial approximations to a monoamine theory come from two nearly simultaneous discoveries in the 1950s – one being the psychotropic effects of reserpine; the other that iproniazid has an antidepressant effect (Baumeister et al., 2003).

Reserpine was isolated as the active component of the dried root *Rauwolfia* (used in India at the time), known to lower BP. Reserpine was shown to have equal anti-hypertensive efficacy as *Rauwolfia* (Wilkins et al., 1954) and subsequently marketed as a sedative-antihypertensive under the trade name Serpasil. Reports from India suggested that *Rauwolfia* was also effective in treating mental disturbances, and subsequently Kline demonstrated its use in schizophrenia in a placebo controlled trial – not to halt the 'schizophrenic process,' but to produce sedation and reduce disruption in wards (Kline, 1954). Although the therapeutic effects associated with reserpine were modest at best, reserpine was used extensively by psychiatrists in the mid-1950s. However its popularity rapidly declined, primarily due to the development of chlorpromazine as a more effective treatment for schizophrenia (Margolis, 1957), but also because of concerns that reserpine caused profound depression in some patients (Harris, 1957).

Iproniazid was first used as an anti-tuberculosis compound in the 1950s following successful clinical trials demonstrating its profound clinical effects, halting the progression of tuberculosis together with reversing apathy and improving patients' sense of well-being (Robitzek et al., 1952). Although initially attributed to improvements in lung function, side effects indicative of a central action of iproniazid (such as dry mouth, constipation and drowsiness) led to speculation that psychological improvements are due to an action in the brain. This triggered clinical trials assessing mood changes following iproniazid administration both in patients with tuberculosis and in psychiatric patients. These trials showed moderate efficacy for iproniazid in improving mood and treating depression: some promising findings (Crane, 1956; Delay et al., 1952; Salzer and Lurie, 1953) were tempered by negative results (Smith, 1953). Widespread introduction of iproniazid into the psychiatrists' toolbox for treating depression is generally attributed with results from a uncontrolled clinical trial by Loomer *et al.* at Rockland State Hospital, New York, who reported improvement in mood in 70% of hospitalized patients treated with iproniazid at time-points between five

weeks and four months following initiation of treatment (Loomer et al., 1957). Following this study, 380,000 patients with depression were treated with iproniazid between 1957 and 1958 (Floody et al., 1958) although its golden age was short-lived: iproniazid was withdrawn in 1961 owing to an association with jaundice.

Despite their short-lived clinical use, studies into the mechanism of action of reserpine and iproniazid were crucial in the development of the monoamine hypothesis of depression. Zeller and Barksy discovered that iproniazid is a potent inhibitor of the enzyme monoamine oxidase (MAO inhibitor, MAOi) (Zeller and Barsky, 1952). The subsequent finding that other MAOis (such as tranylcypromine) also had anti-depressant effects confirmed this as the mechanism of action for mood changes associated with the drug. The chemical similarity of reserpine and 5HT (both indolamines) led to the suggestion that reserpine works through an action on endogenous 5HT and subsequently, reserpine was found to deplete 5HT and the time-course of depletion matched the time-course of reserpine's therapeutic action and negative mood induction (Brodie et al., 1956). Later work revealed reserpine's action more precisely: namely, to deplete catecholamines through its role as an irreversible vesicular monoamine transporter (VMAT) inhibitor (Henry and Scherman, 1989).

The link between the action of these drugs on central amines (iproniazid to enhance and reserpine to deplete) and their psychological effects (iproniazid to enhance mood and the reserpine to reduce mood) led to early formulations of the monoamine hypothesis (Baumeister et al., 2003) as outlined by Everett and Toman:

> *"One may speculate on the possible role of centrally active amines present in the brain in the normal activity and general responsiveness of an individual. An excess of these might result in irritability, restlessness and aggressiveness. In the opposite direction, a deficiency of these substances would result in depressions and general lassitude."* (Everett and Toman, 1959)

Although this was an early instantiation of the monoaminergic theory, it was not until Schildkraut integrated multiple lines of evidence that a compelling biochemical theory of depression was developed (Schildkraut, 1965). Schildkraut's hypothesis asserted that drugs decreasing monoamines result in depression, whereas drugs increasing monoamines decrease depression. The focus towards 5HT (over other neurotransmitters) developed from this, owing to a gradual accumulation of numerous lines of evidence:

- **Tryptophan loading**: In both healthy controls and patients with schizophrenia, ingestion of tryptophan – the precursor of 5HT – produces elevated mood (Oates and Sjoerdsma, 1960; Pollin et al., 1961; Smith and Prockop, 1962). Tryptophan was also

shown to potentiate the antidepressant action of the MAOi tranylcypromine (Coppen et al., 1963).

- **5-hydroxyindoleacetic acid (5HIAA) concentrations in cerebrospinal fluid (CSF)**: Ashcroft and Sharman estimated the concentration of the 5HT breakdown product 5HIAA in human CSF of controls and depressed patients (Ashcroft and Sharman, 1960). They found reduced 5HIAA in the depressed group. Based on this, they reasoned that 5HT concentrations were also decreased.
- **Action of tricyclic antidepressants (TCA)**: Insight into the molecular mechanisms behind the action of the prototypical TCA imipramine on mood further bolstered the serotonergic version of the monoamine hypothesis. Marshall and colleagues discovered that imipramine blocked 5HT reuptake into platelets (Marshall et al., 1960).

These converging lines of evidence led Coppen to propose the 5HT version of the monoamine hypothesis (Coppen, 1967): depression is the result of low levels of central 5HT. This hypothesis gained momentum following the success of Selective Serotonin Reuptake Inhibitors (SSRIs) as first-line therapies for depression, together with the effect of acute tryptophan depletion to lower mood (Young et al., 1985) and induce depression relapse (Leyton et al., 2000). However, the role of catecholamines such as NA in depression was not neglected: imipramine was also shown to block NA reuptake in pre-synaptic terminals (Axelrod, 1964) and Serotonin-noradrenaline reuptake inhibitors (SNRIs) such as venlafaxine and duloxetine have also been developed as effective antidepressant agents. Since these initial proposals, the relative contribution of reduced 5HT *vs.* reduced NA to the depressive phenotype has been debated. TCAs such as imipramine and clomipramine act as SNRIs by blocking the reuptake transporters for both NA and 5HT approximately equally (with minimal effect on the DA reuptake transporter). It is possible that reductions in 5HT and NA are linked to different aspects of depression – drugs that increase NA appear to be better at improving symptoms of apathy, anhedonia and fatigue, whereas drugs that increase 5HT tend to elevate mood and improve ‘painful’ symptoms of depression such as guilt and distress (Moret and Briley, 2011; Nutt, 2008; Nutt et al., 2007).

The monoamine theory of depression – in its various forms – has stimulated vast amounts of informative research regarding the neurobiological and neurochemical mechanisms of depressive disorders. At its heart, however, the idea behind the monoamine/5HT theory is correlative: reduced monoamine levels are correlated with low mood, and increased monoamine levels are correlated with the therapeutic effects of antidepressants (such as MAOis and TCAs) – therefore low monoamine availability is the cause of low mood and depression.

Additionally, the theory struggles to explain several features of SSRI/SNRI action:

- **Moderate efficacy of monoamine-based antidepressants:** First-line treatment with antidepressant drugs which target the monoamine systems achieve remission rates of 50% over 12 weeks of treatment (Rush et al., 2006a).
- **Time-lag in drug action:** There is a temporal discrepancy between the immediate effects of SSRI/SNRIs on the availability of monoamines at synapses (within minutes/hours) and the therapeutic effects of the drugs (3 weeks or longer) (Heninger et al., 1996; Hyman and Nestler, 1996). Multiple delayed pharmacological effects of antidepressants have been identified which could explain the time-lag (Frazer and Benmansour, 2002) – including effects on second-messenger systems (Popoli et al., 2000) and on neural plasticity (Duman et al., 1999) – suggesting effects beyond simple increases in monoamine availability.
- **Actions of tianeptine:** A potential problem with the monoamine hypothesis is related to the action of tianeptine, which may treat depression through its action as a selective serotonin reuptake enhancer (SSRE) (McEwen et al., 2010). Its role as an SSRE is directly opposite to SSRIs but its efficacy is well documented (Kasper and McEwen, 2008). However, whether tianeptine truly is an SSRE is not clear, as it fails to increase or decrease 5HT levels in the cortex of conscious rats and does not appear to have any measurable sustained effects on 5HT handling (McEwen et al., 2010). The antidepressant effects of tianeptine may be through long-term neuroplastic and structural changes in neurons of structures within the limbic system. Recently, tianeptine has been shown to be an agonist at the μ-opioid receptor, with opiate-like properties of analgesia and reward without tolerance and withdrawal (Samuels et al., 2017).

Therefore, whilst the monoamine theory does carry explanatory weight, (i) changes in monoaminergic neurotransmission alone are unlikely to account for all cases of depression and (ii) the antidepressant actions of monoamine-based therapies may be related to downstream, neuroplastic changes rather than acute effects associated with short-term increases in monoamine concentrations.

1.5.1.4.6 Dysfunction within the glutamatergic system and neuroplastic changes

The glutamate theory of depression suggests that the pathophysiology of depression is caused by malfunction in mechanisms regulating the central clearance and metabolism of glutamate. This results in structural and functional changes in multiple brain areas including those involved in the regulation of emotion, cognition and visceromotor function (Sanacora et al., 2012). The complexity and variety of changes that can be induced from insults to the

glutamatergic system may explain (in part) the array of behavioural and psychological symptoms of depression which can vary widely between individuals.

The glutamate hypothesis formed based on a similar line of reasoning to the monoamine theory: drugs which act on the glutamatergic system can have antidepressant effects. For example, NMDA receptor antagonists (such as AP-7 and MK-801) exert an antidepressant action in rodent inescapable stress models of depression (Trullas and Skolnick, 1990). The subsequent emergence of a glutamate theory of depression was largely due to its role as the major excitatory neurotransmitter in the central nervous system (CNS) (Orrego and Villanueva, 1993). In the neocortex, 85% of all synapses are glutamatergic (Douglas and Martin, 2007), and glutamate is therefore significantly more prevalent than other neurotransmitters in the brain (including 5HT, NA, DA and – to a lesser extent – gamma-aminobutyric acid, GABA). Indeed, the multi-faceted role of the monoamine neurotransmitters in sleep, motivation, emotion and cognition may ultimately depend on changes in excitatory glutamatergic neurotransmission (and its balance with inhibitory GABAergic tone). There are multiple lines of clinical evidence suggesting glutamatergic dysfunction in mood disorders:

- **Changes in peripheral and central glutamate levels from blood and tissue samples**. Elevated glutamate content has been observed in depressed individuals' plasma compared to controls (Altamura et al., 1995; Kim et al., 1982) Within the CNS, post-mortem samples from unipolar and bipolar depressed patients indicate increased glutamate levels in the frontal cortex (Hashimoto et al., 2007).
- **Changes in central glutamate levels from *in vivo* magnetic resonance spectroscopy (MRS) studies.** *In vivo* MRS facilitates the assessment of glutamate levels in targeted neuroanatomical regions, and avoids the practical difficulties associated with post-mortem tissue collection. However, it is difficult to assign a resonance peak to glutamate exclusively so a combined measure term (Glx) is calculated which predominantly reflects glutamate levels but also contains glutamine. Since the first study demonstrating an association between mood and Glx measures (decreased Glx correlated with a single patient's transient experience of suicidal depression) (Cousins and Harper, 1996) there have been multiple studies examining the relationship between glutamate and its related metabolites, and mood. The results are variable, although trends have emerged from the literature including (i) reduced Glx in the frontal/cingulate regions in patients with major depressive disorder (MDD) in the midst of an episode (Auer et al., 2000; Hasler et al., 2007a; Michael et al., 2003); (ii) elevated Glx in the parietal-occipital regions in patients in an acute depressive episode (Sanacora et al., 2004), in remission (Bhagwagar et al., 2005)

and in at risk groups (Taylor et al., 2011); and (iii) increased Glx in the frontal cortex of elderly patients with MDD (Binesh et al., 2004) and post-stroke depression (Glodzik-Sobanska et al., 2006; Wang et al., 2012). Overall, the Glx values seem to vary significantly by brain region, subtype of mood disorder and age of onset.

- **Changes in glial cell function**. Astrocytes are critically involved in the regulation of glutamate levels in the brain through the glutamine-glutamate cycle. Reduced glial cell density has been repeatedly reported in depression (Cotter et al., 2001; Ongür et al., 1998b; Rajkowska et al., 1999). Importantly, reduced expression of the excitatory amino acid transporters (EAAT1, EAAT2) – located on astrocytes – has been identified in depressed patients (Bernard et al., 2011; Choudary et al., 2005a; Miguel-Hidalgo et al., 2010; Sequeira et al., 2009) which would result in impaired glutamate clearance from the synaptic space and dysfunctional glutamate/glutamine cycling. These findings are highly relevant to the experiments in this thesis, as the principle drug used to over-activate subregions of the vmPFC is dihydrokainic acid (DHK, used in **Chapter 3**, **Chapter 4** and **Chapter 5**) – an EAAT2 inhibitor.
- **Changes in glutamatergic synapses following stress.** Stress and inappropriate or excessive activation of the HPA axis is associated with low mood and the development of depression (see **1.5.1.4.4**). Acute stress seems to induce glutamate release in the hippocampus, amygdala and prefrontal cortex through rapid non-genomic mechanisms (see (Sanacora et al., 2012)), although the impact of chronic stress is less well characterized. Direct administration of cortisol has also been shown to increase membrane trafficking of AMPA receptor subunits (Groc et al., 2008), and stress paradigms increase activity within NMDA and AMPA receptor mediated circuits in rat pyramidal neurons (Yuen et al., 2009). These lines of evidence suggest that the effects of stress – including deleterious effects on mood – may be mediated at least in part by alterations in glutamatergic neurotransmission.
- **The influence of antidepressants on the glutamate system**. Antidepressants appear to modulate NMDA receptor function (Paul et al., 1994) and influence the expression of NMDA receptor subunits at synapses (Boyer et al., 1998). In so doing, these drugs influence glutamate-mediated functions such as LTP, although the magnitude and direction of effects are somewhat mixed and vary depending on brain area (Pittenger and Duman, 2008). Amongst the most consistent effects are reports of reduced hippocampal LTP after both acute (Kojima et al., 2003) and chronic (Stewart and Reid, 2000) antidepressant treatment.

  In addition to influencing the function of glutamate receptors, a number of studies have also found that antidepressants attenuate the release of glutamate in the frontal cortex and hippocampus (Bonanno et al., 2005; Tokarski et al., 2008). This effect to

reduce glutamatergic tone has led to the suggestion that antidepressants limit the excess of glutamate overflow induced by acute/chronic stress. This has been corroborated by animal studies: for example, tianeptine abolishes increases in extracellular glutamate induced in the amygdala following restraint stress (Reznikov et al., 2007).

Excitingly, pharmacological agents with a direct action on the NMDA receptor have shown promise as highly effective antidepressants. The prototypical glutamate-based antidepressant is ketamine, an NMDA receptor antagonist. Multiple clinical trials have illustrated the rapid and relatively sustained effects of one-off ketamine doses in treating symptoms of depression (Berman et al., 2000; Diazgranados et al., 2010; Liebrenz et al., 2007; Murrough et al., 2013a, 2013b; Valentine et al., 2011). The beneficial effects of ketamine have also been observed in a number of animal studies (Autry et al., 2011; Du et al., 2006; Garcia et al., 2009; Koike et al., 2011; Maeng et al., 2008).

The glutamate hypothesis has evolved to incorporate multiple lines of evidence which suggest changes to synaptic function, altered synaptic plasticity and circuit-level remodelling are cardinal features of depression: this is termed the neuroplasticity hypothesis (Pittenger and Duman, 2008). The hypothesis asserts that (i) plastic changes induced by glutamatergic transmission can be adaptive (plastic changes induced by learning and exercise) as well as maladaptive (plastic changes associated with mood disorders) and (ii) therapeutic treatments (such as antidepressants) can reverse maladaptive plastic changes (Bessa et al., 2009; Hajszan et al., 2009; Norrholm and Ouimet, 2001).

The majority of neuroplastic changes associated with depression take place within the glutamatergic system (Sanacora et al., 2012). Simply by virtue of its prevalence, glutamatergic transmission is crucial in the generation and regulation of emotion. Accumulating evidence suggests that there are neuroplastic changes in prefrontal cortico-limbic circuits mediating aspects of emotion that are responsible (at least in part) for symptoms of depression. In particular, stress-related animal models of depression show that stress can cause widespread changes in the glutamate system together with structural-morphological changes in the synapses and dendrites of prefrontal cortical neurons (Holmes and Wellman, 2009; McEwen, 2005; Shansky and Morrison, 2009). These widespread structural changes can result in changes in gross morphology, potentially explaining volumetric changes observed in post-mortem imaging studies of depressed brains (Koolschijn et al., 2009; Lorenzetti et al., 2009).

Glutamatergic neurotransmission undoubtedly interacts with the monoaminergic systems (Pralong et al., 2002), but given its critical role in regulating emotion and cognition and its

abundance as a neurotransmitter, several researchers have called for a greater focus on the glutamate system as a final common pathway for depressive symptoms and therapeutic treatments. This shift in focus may have therapeutic implications: there are no approved antidepressant agents directly targeting the glutamate system, but less than one-third of depressed patients achieve remission with current monoamine-based therapies (Trivedi et al., 2006). Glutamate-based strategies therefore represent a novel avenue to be explored for more effective antidepressants.

1.5.1.4.7 Dysfunction within the endogenous opioid system

Preclinical evidence suggests that activation of opioid receptors has antidepressant-like effects, with agonists decreasing immobility time in the tail-suspension test (Berrocoso et al., 2013). The effects of these agonists are blocked by the non-selective opioid-receptor antagonist naloxone (Zomkowski et al., 2005). However, this evidence does not causally implicate reduced opioid transmission in depression-like behaviours. Indeed, the non-selective opioid receptor antagonist naloxone has no effect on the forced swim test, but does diminish the efficacy of TCAs to reduce immobility time (Devoize et al., 1984) suggesting that these antidepressants exert some of their effects by modulating opioid transmission. The lack of effect of naloxone on these tests may relate to its lack of selectivity as an antagonist, as the different classes of opioid receptor are proposed to have different functions.

The relevance of different opioid receptor subtypes, including the μ-opioid receptor, δ-opioid receptor and κ-opioid receptor, remains unclear. For instance, whilst some have suggested that the μ- and δ-subtypes exhibit antidepressant effects, whereas activation at the κ-subtype induces depressive-like states (Peciña et al., 2018). However, mice with μ-receptor knockouts exhibit decreased anxiety and depressive-like behaviours, δ-receptor knockouts exhibit the opposite pattern and κ-receptor knockouts have no effect (Lutz and Kieffer, 2013). More preclinical work is necessary to comprehensively disentangle the roles of different opioid receptors.

Evidence in humans supports a role for opioid transmission in MDD. Opioid agonists such as morphine and heroin are euphorigenic, and opioid receptors are prevalent in limbic brain structures suggesting that opioid transmission is important in mood (Lutz and Kieffer, 2013). Opioid neurotransmission has been linked to emotional resilience and reduced negative affect (Hsu et al., 2013), and attenuated functioning of the endogenous opioid system has been observed in depressed patients during social rejection situations which require emotion regulation (Hsu et al., 2015).

Clinically, 51% of all opioid prescriptions for pain are for the 16% of Americans who suffer from comorbid depression and anxiety (Davis et al., 2017), and substance abuse disorders –

including opioid abuse – are highly comorbid with depression, leading to the suggestion that patients with mood disorders may be using opioid agonists to 'self-medicate' for the symptoms of their disorder in these instances (Markou et al., 1998; Peciña et al., 2018). Furthermore, substance use disorders (including opioid abuse) The use of opioid agonists to treat symptoms of depression dates back hundreds of years, although the risks of abuse and overdose have limited their therapeutic potential. Nevertheless, the effects of intravenous infusions of endorphin peptide preparations have been evaluated, showing substantial improvements of depressive symptoms within hours of administration (Gerner et al., 1980; Kline et al., 1977; Pickar et al., 1981). More recently, the partial μ-opioid receptor agonist buprenorphine has been extensively investigated – its partial agonism properties having safety advantages and reducing the likelihood of abuse. Its antidepressant action may also, in part, be contributed to by antagonism at the κ-opioid receptor (Carlezon et al., 2009). Both intravenous (Emrich et al., 1982) and sublingual (Bershad et al., 2018) administration of low-dose, sub-euphoric buprenorphine seems to have therapeutic potential in the treatment of depression. Low-dose buprenorphine also reduces suicidality in acutely suicidal patients (Yovell et al., 2015). Therefore, the opioid system represents a promising therapeutic target for the treatment of depression.

1.5.1.5 Treatment of Depression

Treatment goals include: eradicating symptoms; minimizing impact of symptoms on daily functioning and quality of life; reducing suicidality; minimizing treatment side-effects; and preventing relapse. Treatment is multi-faceted and nuanced, including medication, psychotherapy, and supportive interventions, together with more interventional measures such as electroconvulsive therapy (ECT) and DBS. Evidence emphasises the importance of integrated, collaborative care (including a multi-professional approach, a structured management plan, scheduled follow-up and communication between service providers) which improves depressive and anxiety symptoms (together with increasing medication compliance when compared to standard care) with benefits sustained for up to 2 years (Cochrane Clinical Answers, 2014).

1.5.1.5.1 Psychotherapy

Psychotherapy is recommended for all patients with depression. Therapists utilise a combination of cognitive behavioural therapy (CBT) (Cuijpers et al., 2013), interpersonal psychotherapy (IPT) (Cuijpers et al., 2011) and problem-solving therapy (Bell and D'Zurilla, 2009). CBT seems to be particularly effective, having an enduring effect to reduce risk of relapse after treatment ends (Hollon et al., 2005). Data from clinical trials suggest a synergistic effect of CBT with antidepressant therapies in treatment-resistant depression (Wiles et al., 2013).

Psychological interventions such as CBT and IPT are associated with neurobiological changes. Two of the most consistent findings in this regard is that a hypometabolic dlPFC shows increases in activity following successful psychological intervention whereas a hypermetabolic sgACC/25 shows decreases in activity (Mayberg et al., 1999).

1.5.1.5.2 Pharmacotherapy for depression: first-, second- and third-generation antidepressants[2]

Treatment with second-generation or third-generation antidepressants is considered first-line therapy for moderate-severe depression. Antidepressant medications are superior to placebo in the treatment of depression (Cipriani et al., 2018; Gibbons et al., 2012). Whilst some studies show no robust differences between different antidepressants in their safety profiles or efficacy (Gartlehner et al., 2011), recent work suggests that there may be important variability in efficacy and acceptability (Cipriani et al., 2018). More severe cases of depression seem to benefit more from antidepressant therapy (Calati et al., 2013; Kirsch et al., 2008). Whilst the causal mechanisms are unclear, the first few weeks of SSRI use are associated with an increased risk of suicidal thoughts in patients under 25 years of age (Gunnell et al., 2005; Miller et al., 2014a; Saperia et al., 2006), although the ultimate effect of successful treatment with SSRIs is to reduce suicidal ideation (Rucci et al., 2011).

TCA monotherapy (first-generation antidepressants such as amitriptyline, desipramine, imipramine, nortriptyline) is generally considered a second-line treatment option. TCAs inhibit both 5HT and NA reuptake and have more side effects compared to their SSRI counterparts, and so are less frequently used in clinical settings. MAOis are considered third-line choices and are rarely used. MAOis interact with many other drugs and food types and are counter-indicated in patients with hypertension (see *BMJ Best Practice: Depression in adults*, https://bestpractice.bmj.com/topics/en-gb/55).

Combination therapy is also possible, and augmentation strategies have been shown to improve antidepressant response; atypical antipsychotic augmentation has the most extensive evidence base (Spielmans et al., 2013). Bupropion – a noradrenaline-dopamine reuptake inhibitor – can work synergistically with SSRIs to enhance the antidepressant response and reduce side-effects (Zisook et al., 2006). Combination therapy with mirtazapine may be beneficial for treatment-refractory patients, although side effects are evident at doses

[2] The terms ‘first-generation antidepressant,’ ‘second-generation antidepressant’ and ‘third-generation antidepressant’ refer to the approximate era of introduction, rather than any similarity in chemical structure or mechanism of action. First-generation antidepressants were introduced in the 1950s and 1960s, and predominantly include tricyclic antidepressants (TCA) such as imipramine, together with iproniazid and isoniazid. Second-generation antidepressants were introduced in the 1970s and 1980s and include bupropion, tianeptine and amineptine. Third-generation antidepressants were introduced in the 1990s and 2000s, and include SSRIs such as fluoxetine, citalopram and sertraline together with SNRI antidepressants such as venlafaxine and duloxetine.

that have been tested (Carpenter et al., 1999). There is also some evidence for lithium augmentation strategies, but there are inconsistencies across studies (see (Fleurence et al., 2009)).

1.5.1.5.3 Pharmacotherapy for depression: novel antidepressants

The use of antidepressant treatments such as those mentioned above has transformed the practice of psychiatry and provided relief to millions of patients. However, these medications are partially effective: only 37% of patients achieve remission following 12 weeks of citalopram treatment, suggesting that up to two-thirds of patients will remain ill despite first-line antidepressant therapy (Murrough, 2012; Rush et al., 2006b). Even for patients who do eventually respond, there is a protracted delay of $\geq$3 weeks before current antidepressants start to work (Machado-Vieira et al., 2010). Patients who fail to respond to multiple first-line therapies are considered treatment resistant and will have higher symptom burden together with a more chronic illness course.

The shortfalls of current therapy have prompted a drive to develop new antidepressants. The NMDA receptor antagonist ketamine has shown significant promise as a rapidly acting, potent antidepressant effective in treatment-resistant populations. The impetus to trial ketamine as an antidepressant resulted from a number of drivers: (i) an implication of glutamate in the pathophysiology of depression (see **1.5.1.4.6**); (ii) the efficacy of NMDA receptor antagonists in animal models of depression (Layer et al., 1995; Trullas and Skolnick, 1990); (iii) the effect of antidepressants on NMDA receptor function; and (iv) preliminary data suggesting efficacy of weak NMDA receptor antagonists in depression (such as amantadine) (Vale et al., 1971).

An antidepressant response associated with ketamine was first reported in 1998 in the context of eating disorder treatment (Mills et al., 1998). Patients received between two and nine ketamine infusions at intervals of five days to three weeks. As well as reducing compulsion scores, administration of ketamine significantly reduced eating disorder-associated negative mood changes. The first placebo-controlled, double-blinded trial to assess the effects of a single dose of ketamine in depressed patients came two years later in 2000, where seven depressed subjects underwent IV infusions with either ketamine (at a low dose of 0.5mg/kg, over 40 minutes) or saline (Berman et al., 2000). Compared to placebo, subjects infused with ketamine showed a rapid yet modest reduction in depressive symptoms (measured by the Hamilton Depression Rating Scale, HDRS) four hours after infusion, with a larger antidepressant response building over 24-72 hours later (**FIGURE 1-21**). The authors noted that the antidepressant response seems "*temporally disconnected from the ketamine-induced euphoria*," highlighting that the depressant response is maximum between one and

three days after infusion, whereas feelings of being 'high' returned to baseline after a few hours.

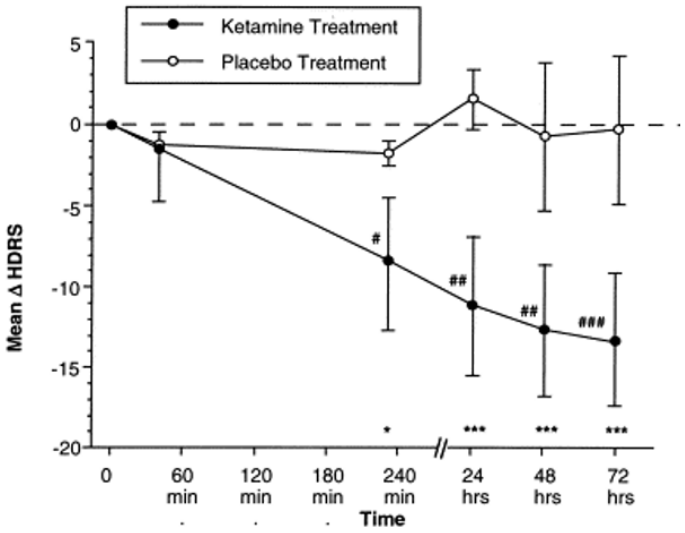


Figure 1-21 Efficacy of a single IV infusion of ketamine in treating symptoms of depression. Figure adapted from Berman *et al*., 2000. Hamilton Depression Rating Scale (HDRS) scores were measured at 4 hours, 24 hours, 48 hours and 72 hours after a forty-minute IV ketamine infusion. Compared to infusions of placebo (saline vehicle), ketamine produced a modest reduction in HDRS score at 4 hours, but greatly abrogated depressive symptoms 24, 48 and 72 hours later.

Since this initial study, further trials have been conducted assessing the efficacy of ketamine together with determining optimal parameters for its administration. This means systematic reviews have now been carried out to review the acceptability of ketamine (and other glutamate-based therapies) in the treatment of depression. One such review found an effect of ketamine at 24 hours, 72 hours and one week after infusion but not at two weeks (Caddy et al., 2015) – however, no significant results were found with other glutamate receptor modulators.

Clinically, different routes of administration have also been tested. Typically, ketamine has been given intravenously at a dose of 0.5mg/kg in 50-100ml of normal saline over 40 minutes (Rao and Andrade, 2010). Two studies have also reported efficacy of intramuscular ketamine in depression (Chilukuri et al., 2014; Harihar et al., 2013), also demonstrating a rapid response within hours. To improve patients' experience of ketamine therapy, oral and intranasal administration has been investigated. For example, a recent retrospective review looking at long-term oral ketamine treatment for depression has demonstrated a 70% reduction in inpatient hospital days and 65% reduction in admission associated with the therapy, together with minimal adverse events (Hartberg et al., 2017). Other studies have reported more modest benefits following open-label treatment with oral ketamine, indicating a

need for further exploration of optimal therapeutic parameters (such as dose and frequency of administration) (Al Shirawi et al., 2017). A recent randomized control trial of intranasal ketamine in major depressive disorder showed a significant improvement in depressive symptoms 24 hours after nasal spray of ketamine compared to placebo: response criteria were attained by 44% of patients with ketamine spray compared to 6% with placebo (Lapidus et al., 2014). An examination of the results of this study shows that a 30% reduction in depression scores had been achieved only 40 minutes after intranasal ketamine. Other reports suggest an action of intranasal ketamine on depression and anxiety symptoms in under 20 minutes (Opler et al., 2016). Although this time course overlaps with the psychoactive effects of ketamine, patients generally report feeling calmer and less anxious, rather than dissociated. Interestingly, the 'ultra-rapid' efficacy of intranasal ketamine is thought to be due to an effect of the spray on vmPFC subregions which lie directly above the cribriform plate, including sgACC/25 (Opler et al., 2016).

The mechanism of action of ketamine is poorly understood. In vivo at clinical dose ranges, it is a selective NDMA receptor antagonist but it has effects on many other receptors (Tyler et al., 2017) – although these effects are weaker than its action at the NMDA receptor (Roth et al., 2013). Several hypotheses have been proposed regarding ketamine's antidepressant action:

- **Disinhibition hypothesis**. Despite being an NMDA receptor antagonist, in 1997 it was reported that ketamine increases prefrontal activity in healthy volunteers (Breier et al., 1997) thought to be due to NDMA receptor inhibition on GABAergic interneurons (Zanos and Gould, 2018). Interestingly the NMDA receptor antagonist MK-801 has been shown to inhibit fast-spiking interneurons in the prefrontal cortex, with a resultant increase in pyramidal neuron firing (Homayoun and Moghaddam, 2007). At latencies similar to its antidepressant action, ketamine enhances gamma-band electroencephalography power which is thought to be related to cortical disinhibition (Pinault, 2008). However, disinhibition is unlikely to fully explain ketamine's antidepressant action because mice lacking (GluN1-containing) NMDA receptors on parvalbumin-expressing inhibitory interneurons retain ketamine's antidepressant effect (Pozzi et al., 2014).
- **Inhibition of spontaneous NMDA receptor-mediated transmission**. Spontaneous release of glutamate occurs at rest as vesicles randomly fuse with the presynaptic terminal membrane, leading to miniature excitatory post-synaptic potentials (mEPSPs). Ketamine and MK-801 have been shown to block NMDA receptor-mediated mEPSPs at rest, leading to inactivation of CaMKIII and de-suppression of

BDNF expression via reduced eEF2 phosphorylation – and the antidepressant effects depend on this rapid upregulation of BDNF (Autry et al., 2011).

- **Inhibition of extra-synaptic NMDA receptors**. GluN2B-containing heterotetrameric NMDA receptors are the principal NMDA receptor subtype located outside of synaptic densities. These receptors are not activated by transient glutamate-dependent neurotransmission within the synapse; instead, they are activated by tonic, low-levels of ambient glutamate in the extracellular space (Rothstein et al., 1996). One hypothesis posits that ketamine acts to inhibit these extra-synaptic NMDA receptors, and it has been shown that GluN2B-containing NMDA receptor knockout markedly reduces the antidepressant effects of ketamine in a mouse model (Miller et al., 2014b). However, in humans, the GluN2B-selective NMDA receptor antagonist traxoprodil does not induce as rapid antidepressant effects as ketamine (Preskorn et al., 2008), and the GluN2B-preferring NMDA receptor antagonist MK-0657 produces modest (and slower) antidepressant benefits (Ibrahim et al., 2011), suggesting that the antidepressant action of ketamine is not solely down to GluN2B-containing NMDA receptor antagonism.
- **NMDA receptor-independent mechanisms**. An important issue with NMDA receptor-dependent theories is that data from clinical trials suggest that alternative NMDA receptor antagonists fail to show the rapid/robust/long-lasting effects of ketamine (Newport et al., 2015). Ketamine is a racemate of (*S*)-ketamine and (*R*)-ketamine, and whilst the former has a greater NMDA receptor affinity, the latter appears to have more potent antidepressant effects in preclinical studies (although both do exert an effect in this regard) (Zanos et al., 2016). To date there are no human trials directly comparing the two enantiomers.
  Importantly, following ketamine administration, the metabolites of the ketamine enantiomers – (*2R,6R*) and (*2S,6S*)-hydroxynorketamine (HNK) – are found in the plasma of mice and humans. Chemically altering ketamine by deuterating the C6 position does not change its affinity for NMDA receptors, but prevents its metabolism into HNK and appears to prevent the antidepressant action of ketamine in mice (Zanos et al., 2016). Furthermore, administration of (*2R,6R*)-HNK has particularly potent antidepressant effects without acting on the NMDA receptor and without any locomotor side effects at effective doses – instead, the efficacy of this metabolite enantiomer seems to be dependent on activation of AMPA receptors (Zanos et al., 2016).

Ketamine has been touted as the most important advance in the treatment of depression in the past half-century (Duman and Aghajanian, 2014). However, knowledge about ketamine treatment is still incomplete and lacking. More data are needed to support the long-term

safety and efficacy of repeated ketamine dosing (including measuring misuse) (Singh et al., 2017), together with its actions at a neurochemical and neurobiological level. Several of the experiments described later in this thesis will address the specific symptom domains in which ketamine is effective, together with the neurobiological substrates involved in its efficacious action (**Chapter 4** and **Chapter 5**).

1.5.1.5.4 Non-invasive neurostimulation: electroconvulsive therapy, magnetic seizure therapy, transcranial direct current stimulation and repetitive transcranial magnetic stimulation

Electroconvulsive therapy (ECT) – involving the electrical induction of seizures – is the oldest neurostimulation therapy for treating treatment resistant depression (Müller et al., 2018). It has shown to be effective (UK ECT Review Group, 2003) and is currently the most common therapeutic option for severe, recurrent depression when medication and psychotherapy has been unsuccessful (Kellner et al., 2012). The neurobiological basis for the efficacy of ECT is unclear, because ECT produces widespread changes in grey matter volume of limbic and paralimbic structures including pgACC/32, sgACC/25 and other regions of vmPFC/ACC (Dukart et al., 2014; Ota et al., 2015; Tendolkar et al., 2013). Magnetic seizure therapy (MST) is a non-invasive convulsive therapy eliciting a generalized tonic-clonic seizure and is being investigated as an alternative to ECT for use during general anaesthesia (where there are added benefits of assisted ventilation and continuous electroencephalography [EEG] monitoring). Although preliminary data suggest that MST may have fewer side effects than ECT, it is still in experimental stages (Allan and Ebmeier, 2011).

Not all neurostimulation techniques induce seizures. Transcranial direct current stimulation (tDCS) involves stimulation of cortical areas using a low-intensity direct current (Palm et al., 2016). Stimulation is focused on left dlPFC with the rationale that this ameliorates the hypoactivity reported in depressed patients (Mayberg, 1997). tDCS is well-tolerated with minimal side-effects, and results in a moderate reduction of depressive symptoms (Meron et al., 2015). A similar, non-seizure inducing therapy, is repetitive transcranial magnetic stimulation (rTMS), involving the external delivery of magnetic pulses to the cortex which induce an electrical potential in brain tissue to depolarize neurons (McClintock et al., 2018). Low frequency rTMS inhibits cortex, whereas high frequency stimulation appears to activate cortex (Bakker et al., 2015). Whilst response rates for rTMS are relatively high at approximately 60%, the associated antidepressant effect is small and transient without maintenance treatment (Kedzior et al., 2015). Current evidence suggests that rTMS could be useful as a treatment strategy alongside other first-line therapies such as pharmacotherapy and psychological therapy, and may be a useful therapeutic avenue to pursue before trying ECT (Perera et al., 2016).

1.5.1.5.5 Invasive neurostimulation: vagal nerve stimulation

Initially developed for epilepsy, vagal nerve stimulation (VNS) involves a minor surgical intervention to implant a pulse generator subcutaneously in the chest, connected to an electrode attached to one of the vagus fibres in the neck (Elliott et al., 2011). Stimulation of the vagus nerve at the point of contact sends impulses in a predominantly retrograde fashion from the periphery to the NST. This modulates activity in a wide array of brainstem and cortical targets including the vmPFC (Müller et al., 2018; Pardo et al., 2008).

Beyond these general effects on neural circuits, the precise mechanism of action of VNS remains largely unknown, and particularly poorly understood is the finding that beneficial effects from VNS tend to accrue over months and years (Nahas et al., 2005). Functional neuroimaging studies have nevertheless provided several insights. Zobel and colleagues found widespread decreases in rCBF after four weeks of VNS, including in pgACC/32 and BA10 (Zobel et al., 2005). Subsequent studies have generally corroborated these findings, showing metabolic changes in sgACC, pgACC and rostral vmPFC regions (Conway et al., 2006; Critchley et al., 2007; Nahas et al., 2007). Pardo and colleagues report that the most significant, extensive change over one year of chronic VNS is baseline hypometabolism localised to the vmPFC, extending from sgACC/25 to rostral BA10 (Pardo et al., 2008).

1.5.1.5.6 Surgical therapies

Surgical interventions are reserved for severe, treatment-resistant cases of depression. Surgical therapy for depression has a long history – early psychosurgical procedures such as the prefrontal leucotomy (involving complete white matter disconnection of large swathes of mPFC, lPFC and OFC) were dangerous and associated with persistent side effects including apathy, anhedonia and a change in personality (Catani et al., 2013). More recently, refined techniques and a more extensive understanding of the neurobiological changes associated with mood disorders has facilitated the development of targeted surgical interventions (Abosch and Cosgrove, 2008; Catani et al., 2013) (**FIGURE 1-22**). These include:

- **Cingulotomy:** This involves bilateral lesions of the ACC and fibres of the cingulum (Cosgrove and Rauch, 2003). Thalamocortical fibres together with efferent projections arising from the anterior cingulate to the amygdala, PAG and NTS are likely destroyed by this intervention. Studies measuring the efficacy of cingulotomies in the treatment of depression report success rates of 40-60% (Ballantine et al., 1987; Spangler et al., 1996).
- **Anterior capsulotomy:** This involves the generation of a lesion in frontothalamic fibres passing through the anterior limb of the internal capsule (medial to the putamen and ventral to the head of the caudate, and immediately dorsal to the accumbens). The primary indication for anterior capsulotomy is obsessive-compulsive disorder

(OCD) (Bingley and Persson, 1978) but it is also used in the treatment of depression (Ridout et al., 2007).

- **Subcaudate tractotomy:** This involves bilateral lesions in the substantia innominata of the basal forebrain, ventral to the head of the caudate. Subcaudate tractotomy has been used to treat treatment-resistant cases of depression, anxiety and OCD for decades (Mashour et al., 2005). Studies have shown significant clinical improvements in over half of depressed patients following subcaudate tractotomy (Göktepe et al., 1975; Poynton et al., 1995).
- **Limbic leucotomy:** Limbic leucotomy is a combination of cingulotomy and subcaudate tractotomy, developed as a means of combining the therapeutic benefit associated with the individual procedures. Of a cohort of six patients, Montoya *et al*. reported three as experiencing significant improvement in symptoms following limbic leucotomy (Montoya et al., 2002).

As discussed, these methods of ablation can benefit a significant proportion of patients with intractable depression. Nevertheless, they are still hampered by problems associated with the presence of an large (2cm) irreversible lesions which permanently impact upon wider brain function (Juckel et al., 2009).

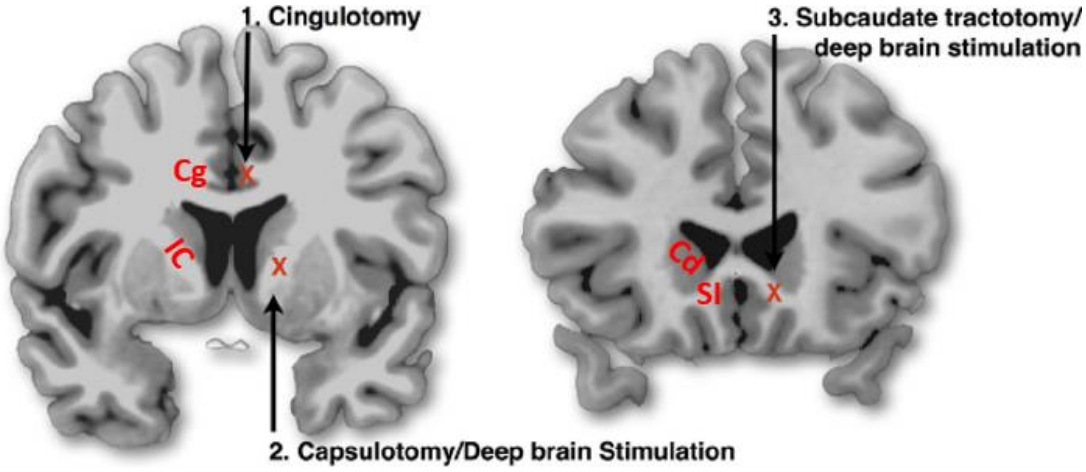


Figure 1-22 Selected neurosurgical interventions for the treatment of depression. Figure adapted from Catani *et al.*, 2013. Cingulotomy (1.) involves bilateral destruction of portions of the cingulate gyrus (labelled Cg; typically encompassing pgACC and dACC) together with white matter tracts forming the cingulate bundle (cingulum). Capsulotomy (2.) involves bilateral destruction of the anterior limb of the internal capsule (labelled IC) immediately dorsal to the caudal portion of the accumbens. Subcaudate tractotomy (3.) involves bilateral substantia innominata (labelled SI) lesions, ventral to the head of the caudate (labelled Cd). A limbic leucotomy is a combination of cingulotomy and subcaudate tractotomy. Also highlighted are novel targets for DBS, including the ventral capsule and subcaudate zone. Not shown is the sgACC/25, which also shows promise as a target for DBS in depression (Mayberg *et al*., 2005).

DBS techniques were developed to better access deeper brain structures for even more focused interventional manipulation of neuronal circuits. First used in neurological settings to treat patients with movement disorders such as Parkinson's disease (Benabid et al., 1987), DBS involves stereotaxic implantation of electrodes into a specific brain region. These electrodes are connected to a subcutaneous generator which provides power and controls stimulation (typically continuous). The electrodes used are only 1.7mm in diameter, facilitating highly specific targeting of neuronal pathways. The first target investigated for DBS of treatment resistant depression was sgACC/25 – Mayberg and colleagues reported alleviation of depressive symptoms in four of six treatment refractory depressed patients following DBS targeting sgACC/25 (Mayberg et al., 2005) (discussed in **vmPFC as a target for treatment**). Other targets have also been investigated, including the ventral capsule/ventral striatum and medial forebrain bundle to the tolerability, safety and potential mechanisms of DBS therapy (Delaloye and Holtzheimer, 2014).

1.5.1.6 Animal models of Depression

Many of the symptoms of depression – sadness, guilt, suicidal ideation *etc.* which rely on self-report measures – cannot be convincingly studied in animals. Developing animal models

is therefore difficult. Nevertheless, such models provide an opportunity to understand molecular, genetic and epigenetic factors which contribute to specific aspects of the aetiology and pathophysiology of depression in cognitive, behavioural and physiological domains.

Animal models of depression should be distinguished from tests. A model represents a state of an organism in which aspects of human pathophysiology are reproduced. A test provides an endpoint – a behavioural or physiological output that can assess the effect of a genetic, pharmacological or environmental manipulation (which themselves may or may not be a model of depression). The merit of both animal models and tests can be assessed based on three criteria for validity. These are used as a benchmark against which animal models and tests of depression can be compared to the disorder being modelled/tested:

- **Face validity**. Does the model/test 'look' like the disorder? Behavioural manifestations in the animal model should resemble symptoms of depression.
- **Construct validity**. Are the same underlying changes involved? Pathophysiological changes occurring in depression – such as neurotransmitter and HPA axis dysfunction – should also mediate changes in the animal model/test.
- **Predictive validity**. Do the same treatments work? Behavioural changes in the animal model/test should be reversed by the same treatments (pharmacological, surgical).

Animal models of depression can be grouped into environmental manipulations (CMS, learned helplessness, maternal deprivation), injuries (olfactory bulbectomy), and chemical manipulations (stimulation of the immune system, psychostimulant withdrawal). Some of these models are particularly powerful – apparently evidencing face, construct and predictive validity. Different models and tests, together with a comparison against validity criteria, are shown in **TABLE 1-8**.

MODEL OR TEST	CRITERION		
	Face	Construct	Predictive
Environmental: Chronic stress (CMS, social isolation)	+	+	+
Environmental: Maternal deprivation	+	+	+
Injury: Olfactory bulbectomy	+	+	+
Chemical: Immune challenge	+	+	+
Test: Forced swim or tail suspension	-	-	+
Test: Sucrose preference	-	-	+

Table 1-8 Animal models/tests of depression against validity criteria. Adapted from Albeira *et al*. (2013).

Of all the animal models of depression, CMS has proved to be one of the most popular. Developed in 1981, CMS typically involves 1-3 months of exposure to a variety of stressors such as cold water immersion, unavoidable shocks and restraint (Katz et al., 1981). Compared to the criteria outlined above:

- **Face validity**: Over the CMS period, animals show neuro-vegetative disturbances such as disrupted sleep and impaired reward processing as measured by blunted sucrose preference (face validity).
- **Construct validity**: Animals show evidence of HPA axis disruption (elevated levels of plasma cortisol), changes in circulating lipid levels and raised levels of inflammatory cytokines (Lucca et al., 2009; You et al., 2011) – evidence of dysfunction within the stress, metabolic and immune systems.
- **Predictive validity**: Changes in animals exposed to CMS are reversed by clinically-effective antidepressants such as TCAs and SSRIs (Szymańska et al., 2009).

Whilst CMS appears to be an appropriate model, it has disadvantages. First, CMS experiments are practically difficult to carry out as they are labour intensive and space-demanding. Second, there is variability in the procedure between laboratories, making replication difficult (Willner, 1997). Third, whilst CMS is useful in assessing depressive pathophysiology, it is not getting to the core of the *aetiology* of the disorder. The same life stressors can lead to depression in some individuals, but not in others. Whilst CMS purportedly exposes animals to 'mild' stress, typical paradigms are intensive and induce pathological changes with a high rate of success. The nature of CMS may be obscuring individual variability in stress-handling and emotional regulation which are so critical in mediating the cause and progression of depression in clinical populations.

Models are only as relevant to psychiatric disorders as the tests use to assess their impacts. Tests such as the forced swim test and tail suspension test are designed to acutely mimic depressive-like behaviour, whereas the sucrose preference test in an assay of reward processing – specifically, reward consumption. Several studies have attested to the predictive validity of these tests: classical antidepressants can reverse impairments on the forced swim test, tail suspension test and sucrose preference tests in animals exposed to CMS (Krishnan and Nestler, 2011; Nestler and Hyman, 2010).

Consider, however, the face validity of these tests. Despite being gold standards for studying depression like behaviours, do tests such as the forced swim and tail suspension tests *look* like the depressed disorder? It has been suggested that immobility represents 'despair' or 'hopelessness.' However it is equally possible that immobility could represent an adaptive learned response to conserve energy when attempts to escape are futile (Anyan and Amir,

2017; de Kloet and Molendijk, 2016). It has also been suggested that rather than immobility reflecting despair/helplessness, the escape-directed mobility phases actually represent enhanced anxiety (Anyan and Amir, 2017).

There are also problems associated with the tests' construct validity. From the clinical literature, it is apparent that the *antidepressant* effects of these drugs take several weeks to develop, and this is at odds with an antidepressant effect of an acute dose as measured in the forced swim/tail suspension test. Germane with the proposal of Anyan and Amir outlined above, the acute efficacy of SSRIs in ameliorating increased immobility times on these tests may be more consistent with their shorter-term anxiogenic effects, acting to stimulate escape behaviours (Silva et al., 1999). Similarly, the reduced sucrose consumption measured on the sucrose preference test is used as an index of impairment in reward processing purportedly consistent with anhedonia (Slattery et al., 2007). However, reward consumption is comparatively unimpaired in depression – changes are more consistently observed in anticipatory and motivational domains (see **1.5.2.4.1**). Therefore, the construct measured in the sucrose preference test is at-odds with the impairments manifesting in patients.

Therefore, whilst there may be animal models of depression which show some degree of predictive, face and construct validity, limitations with the models themselves together with the assays used to assess them currently constrain their utility. Nevertheless, animal models of depression have greatly enriched our understanding of the disorder and provide an avenue from which new treatments can be developed.

1.5.2 Anhedonia: impaired reward processing in depression

Anhedonia – defined as a reduced ability to experience pleasure - is a core feature of MDD, and it is one of the earliest psychopathological symptoms found in clinical descriptions of depression and melancholia (James, 1902). Despite this, the relevance of anhedonia to the depressed state has often been neglected, owing to a predominant focus on the enhanced negative affect associated with depression, but there is a growing appreciation that anhedonia represents a tractable symptom construct which, if treated, could greatly improve patient quality of life.

1.5.2.1 The importance of anhedonia

MDD is a common and debilitating condition, and anhedonia is one of its hallmark symptoms. This is reflected in the Diagnostic and Statistical Manual (DSM)-V criteria for the diagnosing MDD, which requires either depressed mood *or* anhedonia to be present before a diagnosis can be proposed (American Psychiatric Association, 2013). It is also a common symptom, with approximately one-third of MDD sufferers experiencing clinically significant anhedonia (Pelizza and Ferrari, 2009).

In addition to being a common feature of depressive syndromes, anhedonia is prevalent in patients with substance-use disorder, Alzheimer's disease, Parkinson's disease and schizophrenia (Der-Avakian and Markou, 2012). Anhedonia has also been measured as an enduring personality trait ('trait anhedonia') (Blanchard et al., 2001; Keedwell et al., 2005) which is present to a greater extent in at-risk groups for MDD (Gotlib et al., 2010; Liu et al., 2011) and may be a trait marker for MDD (Loas, 1996). Variations in trait anhedonia have neurobiological correlates: higher levels are associated with reduced reactivity of critical reward pathways involving the nucleus accumbens and ventral tegmental area (VTA) (Keller et al., 2013).

In patient groups, presence of anhedonia has important prognostic implications. The presence of anhedonia predicts non-responsiveness to antidepressant therapies (Spijker et al., 2001; Uher et al., 2012) including SSRIs (McMakin et al., 2012) and rTMS (Downar et al., 2014a). Accumulating evidence suggests that conventional antidepressants do little to alleviate anhedonia (Nutt et al., 2007), and may actually contribute to blunting of appetitive behaviour (Hindmarch, 1998; McCabe et al., 2010). Therefore, novel therapies effective in treating anhedonia are sorely needed.

1.5.2.2 Historical accounts of anhedonia

Although the earliest descriptions of anhedonic-like symptoms trace back to the early 19th century (Haslam, 1809), the term '*anhedonie*' was first used in 1896 by Ribot to describe an inability to feel pleasure and withdrawal from pleasurable daily activities (Ribot, 1896). Ribot considered anhedonia in the context of analgesia, striking a contrast between insensitivity to pleasure and insensitivity to pain. However, William James recognized anhedonia as part of 'melancholy' (roughly equivalent to MDD) in 1902 (James, 1902). For several decades, anhedonia received relatively little attention as psychologists focused on the negative emotional aspect of depression ('grief') rather than reductions in positive emotion.

An important development came in 1975, when psychologist Paul Meehl conceptualized anhedonia in a manner closely related to behavioural psychology. Meehl proposed the hedonic capacity model of anhedonia; namely, that hedonic capacity is a trait which varies within the population. 'Hypohedonic' individuals have a perceived weakness of positive reinforcers which usually serve as 'softeners' of aversive states. This weakness is acutely apparent in patients suffering from anhedonia and is associated with two consequences: (i) the normal pleasure associated with goal attainment is not there and therefore behaviours are not reinforced; and (ii) the occurrence of negative internal states such as anger and fear are more frequent. Meehl's recognition of the importance of trait variations in hedonic processing is pithily summarized in the following quote:

"…[some people] are born three drinks behind."

Meehl's characterization of anhedonia was particularly important as it suggests anhedonic deficits can act as a predisposing factor for MDD (as well as other mental health diseases), and that there are 'trait' features of anhedonia which are identifiable before the disease develops. Subsequently, Chapman and colleagues built upon Meehl's characterisation, and distinguished between social and physical aspects of state anhedonia. They defined physical anhedonia as an absence of pleasure derived from physical or sensory experiences (e.g. eating, touching) whereas social anhedonia as an inability to enjoy interpersonal and social pleasures (e.g. talking, being with others) (Chapman et al., 1976).

Klein is widely credited for emphasising anhedonia as a key feature of MDD (Der-Avakian and Markou, 2012; Klein, 1987). Following his account of reward-related deficits in depression, a "loss of interest or pleasure in usual activities" was adopted as a core feature of MDD in the revised edition of the DSM-III (American Psychiatric Association, 1987) and anhedonia was included as a negative symptom of schizophrenia in DSM-IV (American Psychiatric Association, 1994). The WHO's ICD-10 does not explicitly use the term anhedonia but does list a "loss of interest and pleasurable feelings" as a non-essential symptom of depressive episodes.

1.5.2.3 Clinical assessment of anhedonia

Anhedonia is rarely assessed in detail clinically, and when it is, it is measured with self-report questionnaires. An limitation of these questionnaires (as with many other symptoms) concerns inaccuracies an individual's introspection of emotional states, with evidence suggesting that conscious (as well as unconscious) components of emotion are difficult to quantitatively assess (Rømer Thomsen et al., 2015). At some coarse level, however, these questionnaires do provide useful information to guide clinical decision-making and in assessing the impact symptoms have on quality of life. See **FIGURE 1-23** for selected examples of items in anhedonia questionnaires.

The first formal anhedonia questionnaire developed was the Chapman Physical Anhedonia Scale (CPAS) (Chapman et al., 1976) which was designed to measure trait anhedonia – participants are encouraged to describe themselves as they have been "during most of [their] adult life." The CPAS measures several domains of pleasurable experience, including activities/hobbies, sensory experiences and food/drink (**FIGURE 1-23A**). A complimentary scale – the Chapman Social Anhedonia Scale (CSAS) – was developed in parallel focusing on social interactions (in line with Chapman et al.'s distinction between physical *vs.* social anhedonia). Whilst routinely used in clinical populations, psychiatrists have called into question its construct validity as the deficits patients' exhibited in the clinic were not

accurately assessed by the CPAS's items (Germans and Kring, 2000). Slightly later, the Fawcett-Clark Pleasure Scale (FCPS) was developed (Fawcett et al., 1983). The FCPS requires participants to report their current state, thereby making it more suited to assessing disease-associated anhedonia in clinical populations. Using a 5-point Likert scale, the FCPS asks respondents to rate their imagined reactions to pleasurable situations including social activities, sensory experiences and senses of achievement (**FIGURE 1-23B**). Similarly, the shorter Snaith-Hamilton Pleasure Scale (SHaPS) (Snaith et al., 1995) assesses hedonic sensitivity over the past few days and asks participants to definitely agree, agree, disagree or strongly disagree with statements in a 14-item questionnaire regarding hedonic responses to pleasurable situations (such as "I would enjoy looking smart when I have made an effort with my appearance"). A common feature of FCPS and SHaPS is that their items exclusively concern the hedonic impact of reward, and none have items which tap into incentive motivational processes. The CPAS, by contrast, does have items that peripherally assess incentive motivation (e.g. "I have had very little desire to try new kinds of food"), but once again, the overwhelming focus is reward 'liking' rather than anticipatory or motivational processes.

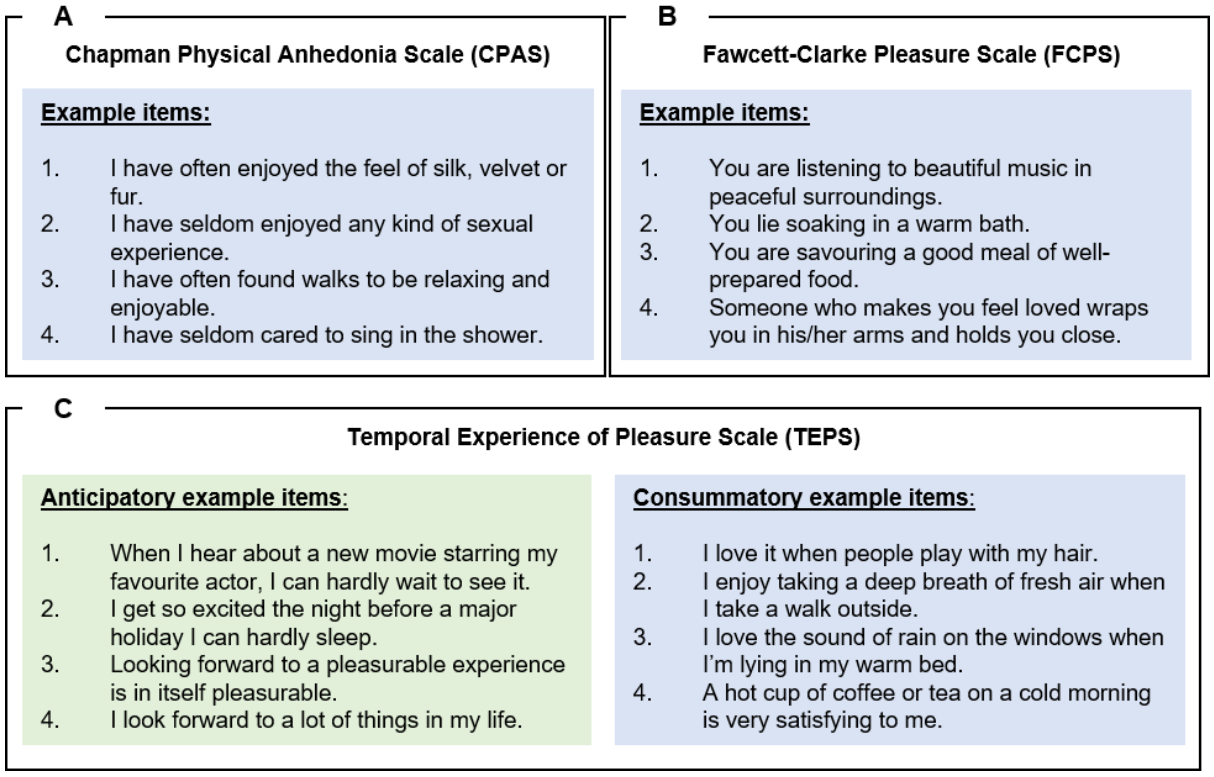


Figure 1-23 Selected items from common anhedonia questionnaires. **A** The Chapman Physical Anhedonia Scale (CPAS) is an interview-based scale consisting of 61 items. These items cover interest in activities and hobbies, sensory experiences, pastimes, social interactions and food/drink. The score is binary, and higher scores are associated with anhedonia. **B** The Fawcett-Clarke Pleasure Scale (FCPS) is an interview-based scale with 36 items covering social activities, sensory

experiences and mastery of difficult tasks. Each item is rated 1-5 on a Likert Scale, and lower scores are associated with anhedonia. Note that the items in the CPAS and FCPS are predominantly concerned with consummatory (hedonic) responses to reward. **C** The Temporal Experience of Pleasure Scale (TEPS) was developed by Gard *et al.* (2006) to distinguish between anticipatory (TEPS-ANT, left) and consummatory (TEPS-CONS, right) pleasure.

To assess reward-related constructs beyond consummatory pleasure, the Temporal Experience of Pleasure Scale (TEPS) was developed (Gard et al., 2006). TEPS distinguishes between anticipatory and consummatory pleasure, with different probes for consumption (CONS, 8 items) and anticipation (ANT, 10 items) (**FIGURE 1-23C**). Using a Likert scale, participants rate items as being "very false for me" to "very true to me." Psychometric analysis of the TEPS scale suggests that the different probes measure distinct constructs: whilst TEPS-ANT is related to reward sensitivity and mental imagery, TEPS-CONS is related to appreciation of positive stimuli ('liking') (Rømer Thomsen et al., 2015). The construct validity of these probes has been studied extensively, particularly in the context of schizophrenia. Gard and colleagues examined correlations between TEPS, CPAS/CSAS and the Behavioural Inhibition/Activation Scales (used to measure appetitive and aversive motivation respectively). The anticipatory components of the TEPS scale had greater correlation with Behavioural Activation Scales reward responsiveness compared to the consummatory components, suggesting a link between diminished reward anticipation and diminished reward motivation. Anticipatory components were also highly related to social functioning assessed by CSAS, implying that reward anticipation is linked to everyday functional status (Gard et al., 2007).

1.5.2.4 Parsing anhedonia

As has been alluded to, although anhedonia is used as a single term to describe reward-related deficits in MDD, these symptoms are not unitary. The definition of anhedonia that has long been in clinical usage focuses on a loss of pleasure (changes in hedonic 'liking'), which can be considered as *consummatory* anhedonia. Largely based on findings from preclinical work into the neurobiological basis of reward-related and motivated behaviours, descriptions of anhedonia have gradually evolved – with an appreciation that it can be fractionated into distinct elements (**FIGURE 1-24**). Beyond consummatory anhedonia, these components include a Pavlovian *anticipatory* anhedonia and an instrumental *motivational* anhedonia ('wanting') (Admon and Pizzagalli, 2015; Der-Avakian and Markou, 2012; Treadway and Zald, 2011). Although separable, the individual components of anhedonia influence one another. For example, impairments in reward anticipation invariably impact on reward

motivation (analogous to the influence of Pavlovian stimuli on instrumental behaviour seen in Pavlovian-to-instrumental Transfer, conditioned reinforcement and conditioned approach).

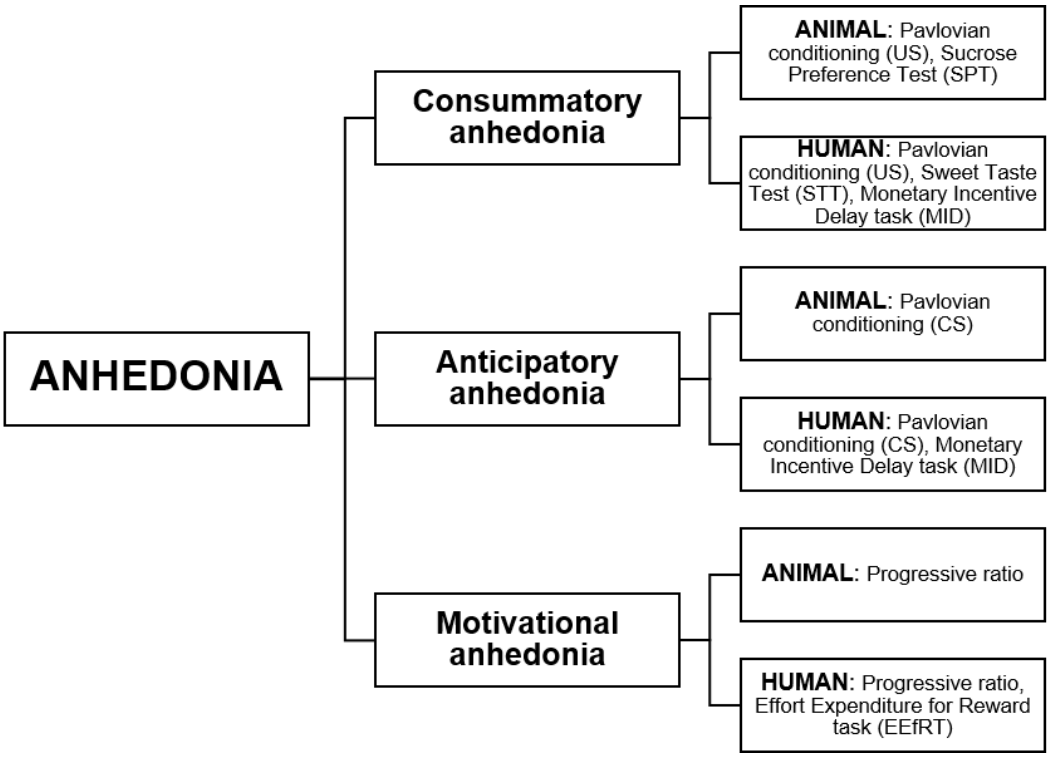


Figure 1-24 Parsing anhedonia. Anhedonia is heterogeneous and can be subdivided into consummatory anhedonia (reduced hedonic capacity), anticipatory anhedonia (impaired arousal responses in anticipation of rewards) and motivational anhedonia (reduced capacity to work for rewards). Shown right are the behavioural assessment tools used in preclinical animal models and in humans to measure the anhedonic impairment or its related reward construct. See text for more detailed discussion of individual tasks.

1.5.2.4.1 Consummatory anhedonia in animal models and patient populations

Consummatory anhedonia refers to a deficit in the primary experience of pleasure – also thought of as reduced hedonic capacity, or reduced reward 'liking.' Consummatory anhedonia has been – and to a large extent, still is – the predominant focus of preclinical and clinical research into anhedonia. For example, items in questionnaires used to assess anhedonia in patient populations almost exclusively focus on the hedonic experience of reward (SHaPS, CPAS, FCPS). Preclinically, the analogue to these scales is the sucrose preference (or consumption) test, where decreased intake of a sweet sucrose solution is argued to reflect an anhedonic state. Rodent models of depression such as CMS reduce sucrose preference (Muscat and Willner, 1992; Strekalova et al., 2011), and manipulations of

rodent PFC which increase depression-like behaviour on assays such as the forced swim test also reduce sucrose preference (Ferenczi et al., 2016; John et al., 2012).

Unfortunately there is a fundamental disconnect between the construct assessed in these studies and the pattern of impairments manifested in depressed patients, who display anhedonic symptoms in anticipatory and motivational domains (Der-Avakian and Markou, 2012; Treadway and Zald, 2011) but relatively intact consummatory responses (Amsterdam et al., 1987; Arrondo et al., 2015a; Berlin et al., 1998; Dichter et al., 2010). This is similar to the pattern of deficits seen in schizophrenic patients (Cohen and Minor, 2010). Furthermore, the anhedonia questionnaires that are widely employed in the clinical domain are only moderately associated with depression severity (Leventhal et al., 2006). These findings suggest that human anhedonia is more than just reduced hedonic capacity.

These data have implications for use of the sucrose preference test as a preclinical model of anhedonia – if consumption is relatively intact in depressed patients, what relevance do reductions in sucrose intake have to the depressed state? The problems for the sucrose preference test do not end there – it has been suggested that overall intake of sucrose is a crude measure even for consummatory hedonic 'liking.' Consider that low and high concentrations of sucrose solution elicit the same amount of consumption despite tasting different and possessing different caloric contents (Dwyer, 2012). This begs the question as to what precisely is driving consumption on the sucrose preference test? The lack of clarity in this regard has led researchers to analyse consummatory behaviours at a microstructural level, with the hypothesis that *how* consumption occurs at a detailed level can provide information about *what* drives it.

One example of this approach is the use of facial reactivity patterns (FRPs) (Grill and Norgren, 1978). FRPs are evolutionarily conserved sequences of facial movements which reliably distinguish between appetitive (sweet) and aversive (bitter) tastes. FRPs are known to reflect the current state of the animal and/or previous experiences of the animal with the particular flavour (Berridge and Schulkin, 1989; Pelchat et al., 1983). The high degree of face validity intrinsic in this task has led to its widespread use in investigating the neurobiology of consummatory anhedonia and hedonics. Extensive 6-hydroxydopamine (6OHDA) lesions of mesolimbic dopaminergic projections to the accumbens and ventromedial caudate fail to reduce FRPs (Berridge et al., 1989). Instead, opioid neurotransmission has been implicated as the crucial hedonic system: Berridge and colleagues have identified subcortical and cortical 'hedonic hotspots' where microinjections of opioid-receptor agonists increase FRPs to positive stimuli. Two such hotspots are the nucleus accumbens (Peciña and Berridge, 2005) and ventral pallidum (Peciña et al., 2006). Conversely, μ opioid receptor antagonism in

– for example – the nucleus accumbens reduces positive FRPs to a sucrose solution (but also affects their incentive motivation for these rewards) (Shin et al., 2010).

Although FRPs have been informative, they are largely qualitative, difficult to analyse in freely-moving animals and scoring is labour intensive. At the same time FRP analysis was being developed as a method to assess hedonics, others focused on developing automated methods to assess lick microstructure in rodents. Rodents free-feeding from a spout of sweet solution lick in clusters (**FIGURE 1-25A**), and the size of a cluster is not random. Instead, the lick cluster size is lawfully determined by the nature of the solution, increasingly monotonically with the concentration of sucrose (Austen et al., 2016; Davis, 1989) (**FIGURE 1-25B**). Interestingly, whilst sucrose-shock pairings affect the amount of sucrose solution consumed but not lick cluster size, pairing sucrose solutions with LiCl (devaluing the sucrose – a true 'taste aversion') affects both sucrose consumption and lick cluster size. Some have suggested that this reflects a distinction between taste *avoidance* (no effect on hedonic value) *vs.* a true taste *aversion* (diminishing hedonic value) (Dwyer, 2012). Broadly speaking, lick cluster sizes are affected by similar manipulations to those that alter FRPs – both decrease with LiCl/sucrose pairings (Baird et al., 2005) and both increase with benzodiazepine/sucrose pairings (Higgs and Cooper, 1998).

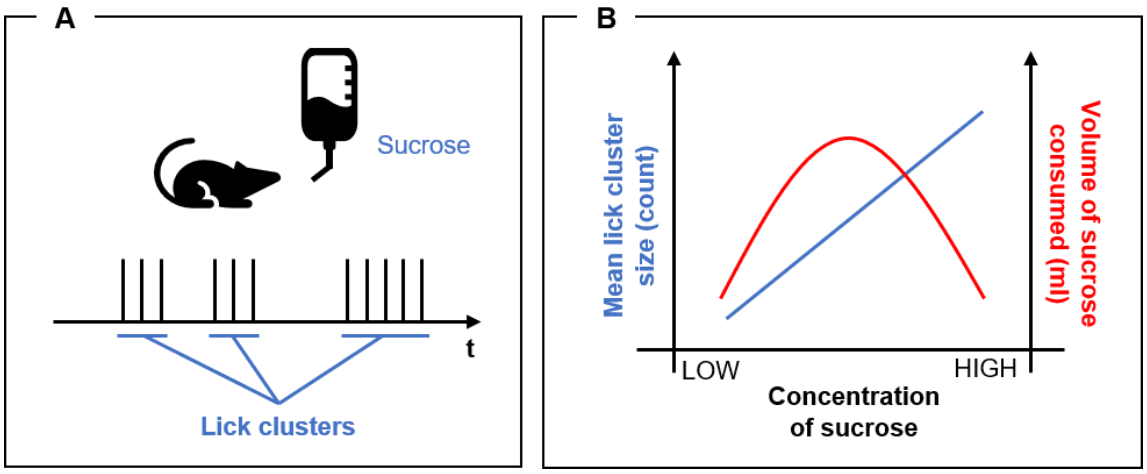


Figure 1-25 Microstructural analysis of consumption. Schematic diagrams based on descriptions in Dwyer, 2012. **A** When consuming solutions from a bottle, rats lick in bouts known as lick clusters. The mean size of lick clusters is not random – it is lawfully determined by the nature of the solution. **B** Absolute sucrose consumption shows an inverted-U pattern (red) such that low and high concentrations of sucrose result in the same amount of consumption. Two very different solutions (both in terms of taste and caloric content) produce the same consumption, meaning it is unclear precisely what feature of the solution is driving consumption. By contrast, lick cluster size is directly proportional to the concentration of sucrose (blue).

Despite their apparent utility in informing us about hedonic processing, very few studies have looked at manipulations which change how pleasurable solutions are consumed – either assessing FRPs or lick cluster size – in the context of depression or models of depression. Preliminary data has suggested that certain psychosocial stress models can reduce lick cluster size (but not the amount of sucrose consumed) together with increasing circulating cortisol levels and attenuating weight gain (Dwyer, 2012). Recently, stressful handling methods such as tail handling have been shown to reduce lick cluster size in mice (Clarkson et al., 2018). However, given an apparent lack of consummatory anhedonia in most depressed patients, one would expect that translationally relevant depression models in animals should leave measurements of consummatory liking unimpaired. Indeed, one study assessing taste reactivity in depressed patients found that depressive symptoms do not appear to alter FRPs to appetitive solutions (Scinska et al., 2004). The importance of the relationship between depression and consummatory anhedonia is explored by experiments in this thesis, showing that manipulations of vmPFC subregions can induce blunted reward processing in anticipatory and motivational domains, whilst leaving reward consumption intact (**Chapter 4**).

In humans, one approach to measuring consummatory liking is the sweet taste test (Dichter et al., 2010). This test yields three types of information: (i) intensity sensitivity to sucrose (slope of intensity ratings *vs.* sucrose concentration); (ii) hedonic sensitivity to sucrose (slope of pleasure ratings *vs.* sucrose concentration); and (iii) whether the participant is 'sweet-liking' or 'sweet-disliking' (does the subject prefer the highest concentration of sucrose solution [sweet-liking] or a lower one [sweet-disliking]). Dichter and colleagues compared depressed patients and controls on each of these metrics. They found no difference in intensity sensitivity, hedonic sensitivity or proportions of sweet-likers/dislikers in depressed patient groups *vs.* controls, providing further evidence that depressed patients are minimally impaired in their hedonic evaluation of rewards.

1.5.2.4.2 Anticipatory anhedonia in animal models and patient populations

The simplest behavioural paradigm that could be used to distinguish between reward anticipation and reward consumption (in both animals and humans) is appetitive Pavlovian conditioning. In this paradigm, reward anticipation can be considered a Pavlovian arousal response to reward-predicting cues (conditioned stimuli, CSs) whereas the arousal response during reward consumption (unconditioned stimuli, USs) reflects hedonic consummatory processing. Arousal during the CS or US could be considered relatively 'pure' measures of anticipation and consumption respectively (although the consummatory period may be contaminated by concomitant vegetative responses during reward ingestion). However,

remarkably few studies have assessed anticipatory/consummatory reward processing in this way in rodents, NHPs and humans. Examples include:

- **Impaired learning of appetitive Pavlovian associations:** In CMS models of depression in rodents, Di Chiara and colleagues have shown impaired appetitive learning together with blunted DA responses in the ventral striatum and PFC (Di Chiara et al., 1999). Impaired appetitive Pavlovian learning has also been demonstrated in depressed patients, together with dysfunction activity in the amygdala, OFC, caudate and pgACC/24,32 (Martin-Soelch et al., 2007). It is important to note that many of these studies assess impairments during learning, so whether these are related to impairments in the affective components of reward anticipation or cognitive learning processes is unclear.
- **Impaired expression of appetitive Pavlovian behaviours:** rats subjected to CMS show reduced anticipatory Pavlovian nose pokes directed to a reward hopper after learning that the hopper is associated with sucrose delivery (Phillips and Barr, 1997). In NHPs, ablative sgACC/25 lesions are associated with a failure to sustain autonomic arousal in a trace interval between the CS and US of a learned Pavlovian association, although (i) CS-induced arousal remained intact and (ii) these effects may result from damage to underlying fibres of passage (Rudebeck et al., 2014). One study looking at neural responses post-learning in humans has shown reduced ventral striatal responses during CSs predicting reward in schizophrenic patients (Dowd and Barch, 2012), with no change in neural activity during reward consumption. No such studies have been carried out in depressed patients. However, in a mixed Pavlovian-instrumental design, Manohar and Husain used a speeded-saccade task with an auditory cue to examine incentivisation by reward-predicting cues (in a manner similar to Pavlovian-to-instrumental transfer) in vmPFC lesioned patients *vs.* controls. They found that vmPFC lesioned patients showed reduced saccadic velocity and autonomic pupillary responses for rewards during cue presentation (Manohar and Husain, 2016).

In **Chapter 4** of this thesis, an appetitive Pavlovian conditioning paradigm is used to examine the causal role of vmPFC over-activity in symptoms of anticipatory/consummatory anhedonia.

Whilst the literature on Pavlovian responses in anhedonic populations is scarce, touchscreen-based tasks have been used to assess reward anticipation in depressed patients *vs.* controls. The most widely used is the Monetary Incentive Delay (MID) task (**FIGURE 1-26**) where (1) an incentive cue is presented (indicating potential gain/loss on the trial), (2) the subject reacts to a target stimulus and (3) the outcome occurs: money is

delivered/money is omitted/loss is avoided/loss is sustained depending on successful/unsuccessful performance (Knutson et al., 2000; Maresh et al., 2014). This well-established paradigm allows the examination of reward anticipation (incentive cue phase) and reward consumption (outcome phase) within a single task. Neurally, several studies indicate that depressed and schizophrenic patients have decreased ventral striatal activity when anticipating rewards in the MID (Arrondo et al., 2015b; Juckel et al., 2006; Nielsen et al., 2012; Stoy et al., 2012).

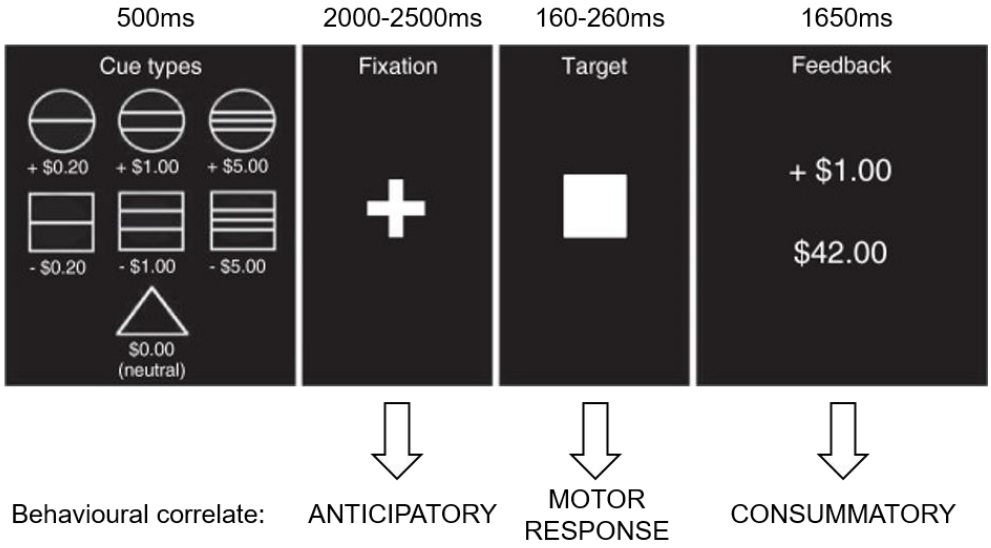


Figure 1-26 Monetary Incentive Delay (MID) task. Task schematic taken from Maresh *et al.*, 2014. Cues indicate the potential to gain (circles) money, lose (squares) money, or a neutral condition (triangle). After the cue, a fixation cross is presented – this period is the anticipatory period. Participants are then presented with a target square when they must press a button as quickly as possible to gain reward/avoid loss. A feedback screen indicates the amount of reward won or lost during the trial, together with the total amount accrued so far. This is the outcome (consummatory) phase. The power of this task lies in its ability to fractionate anticipatory and consummatory aspects of reward processing. Note that the anticipatory phase is confounded by the anticipation to make a motor response.

Several studies have highlighted a role for PFC subregions in reward anticipation measured by MID. Data from Dillon and colleagues suggests that activity within dACC/24 is most closely related to reward anticipation; activity in pgACC/32 is linked to rewarding outcomes; and ventral striatal activity spans both phases (Dillon et al., 2008). Data from other studies suggests there is a role for vmPFC in reward anticipation. Patients with vmPFC lesions (centred around rostral BA10) fail to show increased ventral striatal activity during anticipation of reward on the MID, suggesting that an intact rostral vmPFC is necessary to

sustain the neural correlates of anticipatory arousal (Pujara et al., 2016) and therefore dysfunction within the vmPFC may contribute to symptoms of anticipatory anhedonia.

1.5.2.4.3 Motivational anhedonia in animal models and patient populations

Ever since animals were shown to work for intracranial self-stimulation (ICSS) targeting the medial forebrain bundle (Olds and Milner, 1954), the importance of monoamines in motivated behaviour has been appreciated. Early theories suggested that the neural basis of reward-related behaviours – particularly the *hedonic* (consummatory) components of reward – could be explained by the observations that a wide array of rewarding stimuli resulted in an increase in dopamine (DA) signalling, originating from the VTA (the mesolimbic DA system). This is true of artificial (ICSS, drugs of abuse) and natural (food, sex) reinforcers. Roy Wise suggested that all forms of reward are mediated specifically by the mesolimbic DA system; more explicitly, that independent of their precise mechanism of action, reinforcers act to increase DA transmission in the nucleus accumbens and this is the central component of reward processing and underlies the hedonic value of appetitive outcomes (Wise, 1978, 1982). However, FRPs are convincingly unaffected by both activation (Di Chiara, 2002; Kaczmarek and Kiefer, 2000; Wyvell and Berridge, 2000) and suppression (Fenu et al., 2001; Peciña et al., 1997) of mesolimbic DA transmission. Furthermore, extensive 6-OHDA lesions of the striatum (enough to produce profound aphagia) fail to disrupt taste liking (Berridge and Robinson, 1998). These studies suggest that mesolimbic DA transmission is not directly related to reward consumption.

Integrating several lines of evidence, Berridge and Robinson have re-appraised the role of mesolimbic DA in their *incentive salience hypothesis* based on findings that dopaminergic manipulations of the mesolimbic system in rodents extensively affect appetitive approach behaviours but not taste ‘liking’ (Berridge and Robinson, 2003; Berridge et al., 2009). In their proposal, mesolimbic dopamine is critically important in reward motivation and reward anticipation, but not consumption. The role of DA is to assign a universal motivational currency – ‘incentive salience’ – to transform sensory information into desired incentives. During appetitive Pavlovian conditioning, DA release during CS presentation makes the CS a ‘wanted target’ of motivation, and effectively imbues the CS with the ability to influence instrumental behaviour in several ways:

- **Pavlovian-to-instrumental transfer** (Lovibond, 1983). In appetitive Pavlovian-to-instrumental transfer, presentation of an appetitive CS invigorates instrumental responding for the same rewarding outcome (outcome-specific) but also invigorates instrumental responding for a different rewarding outcome (outcome-general).

- **Conditioned reinforcement** (Hyde, 1976). A CS is a 'conditioned reinforcer' when it can support instrumental responding (e.g. lever pressing) due to a previously learnt association between the CS and a rewarding outcome .
- **Conditioned approach** (Brown and Jenkins, 1968). The process by which a CS acquires reinforcing properties that promote approach towards it (typically studied using 'auto-shaping' preparations). The CS can also elicit consummatory responses that are appropriate to the reinforcer – for example, rats may lick a CS associated with a liquid US.

The importance of DA signalling is not necessarily restricted to discrete CSs: during instrumental conditioning, DA signalling may also attribute contextual cues and manipulanda with motivational significance which act to support ongoing responding.

Parallel perspectives on DA have emerged, predominantly based on electrophysiological studies in macaques carried out by Wolfram Schultz and colleagues. Recordings of midbrain DA neurons in the macaque indicate that phasic firing in these neurons is in accordance with a Rescorla-Wagner 'prediction error' term (Rescorla and Wagner, 1972), such that the release of DA rewarding US is maximal when the reward is unpredicted and minimal when the reward is fully predicted by a CS (Schultz et al., 1997). Schultz's hypothesis posits that rapid phasic changes in mesolimbic DA transmission which show these patterns of firing are critical in reward-based associative learning. Schultz has also emphasised that phasic and tonic levels of DA can change over time-courses that vary over several thousand fold (Schultz, 2000):

- **'Phasic' electrophysiological changes**: A 'short' time-course of milliseconds-seconds. Phasic DA (200ms) represents the reward prediction error signal. A slower, ramping electrophysiological uncertainty signal (2s) has also been measured, whose magnitude is proportional to the degree of variance in reward distribution (maximal when CS predicts US with p=0.5).
- **'Phasic' voltammetric changes**: A 'medium' time-course of hundreds of milliseconds-seconds. Voltammetric changes in [DA] lie on a spectrum – the fastest changes broadly corresponding with the electrophysiological reward prediction signal, and the slower changes corresponding to changes in behavioural outputs.
- **'Phasic' microdialysis changes**: A comparatively 'long' time-course over tens-of-seconds – tens-of-minutes. Changes in [DA] measured by microdialysis are approximately 200-1800x slower than voltammetric changes, and 3000-18000x slower than electrophysiological responses. Because of their longer time-course, they are difficult to relate to discrete events. Changes in microdialysis [DA] are thought to

be related to slower, underlying behavioural processes related to appetite, hunger and satiation.

- **'Tonic', no temporal change (steady-state DA)**: In DA lesioned animals, many behaviours (such as profound aphagia/adipsia) are rescued by administration of a DA agonist and therefore do not require temporal fluctuations in [DA]. The mere presence of DA enables many different behaviours, so steady-state DA may serve a tonic enabling function.

The interpretation of the function of DA at different time-courses is not straight-forward. For instance, temporal changes in DA may not be necessary for reward associations: even after near-total DA lesions, some new values for sweet rewards can be learnt, suggesting that associative representations are 'outside' of mesolimbic DA transmission and consequently that DA signalling is not necessary for reward-based learning (Berridge and Robinson, 1998). Nevertheless, both the Berridge-Robinson and Schultz perspectives suggest DA has a critical role in reward-based motivation, either through rapid phasic transmission mediating incentive salience and/or associative learning, slower changes reflecting motivational state, or a combination of both.

It is therefore, perhaps, unsurprising that manipulations and lesions of the DA system can induce profound motivational deficits akin to motivational anhedonia. Animal models suggest a pivotal role for DA in reward motivation as indexed by overcoming response cost: animals with DA-depleting lesions in the accumbens prefer low-cost/low-reward options, whereas control animals prefer high-cost/high-reward options $\geq$90% of the time (Salamone et al., 2007), suggesting that experimentally-induced increases in low-cost/low-reward choices are pathological in nature (and therefore represent a model of motivational anhedonia) (Treadway and Zald, 2011).

The progressive ratio task has also been used to assess reward motivation – together with the consequences of DA disruption – in animals. In progressive ratio paradigms, animals make an instrumental response to obtain reward under (typically exponentially) increasing response demands (Hodos, 1961). Eventually, the cost of responding is too high for the reward offered, and the animal stops responding. This is termed the breakpoint. Psychostimulant withdrawal (a model of depression which results in 'anhedonia') (Markou et al., 1998) depletes accumbens DA (Weiss et al., 1992) and increases avolition on progressive ratio schedules as measured by reduced breakpoints (Harrison et al., 2001; Stoker and Markou, 2011). In mice, D1 receptor knockout attenuates progressive ratio responding, without affecting sucrose consumption (El-Ghundi et al., 2003) providing further evidence for a neurobiological dissociation between reward wanting and liking.

The role of prefrontal regions in progressive ratio responding has seldom been explored, although some rodent studies have pointed to a potential role for (vm)PFC dysfunction in motivational anhedonia. In 1995, McGregor and Roberts found that injections of the D1 receptor antagonist SCH-23390 into IL decreased breakpoints when rats responded for intravenous cocaine on a self-administration schedule of reinforcement (McGregor and Roberts, 1995). However, Walton and colleagues found that whilst lesions of AC cause a preference for low-cost/low-reward options, combined lesions of PL and IL had no effect (Walton et al., 2003). An evident caveat of this study is the nature of the lesion, affecting both PL and IL (which may have distinct or even opposing roles in behaviour). Supporting the hypothesis that individual sectors of rodent vmPFC have distinct roles, Gourley and colleagues have found that if PL lesions are given *before* mice learn to escalate response requirements on a progressive ratio schedule, they impair performance. However, if mice have learnt progressive ratio parameters prior to lesion surgery and are then lesioned, PL lesions have no effect (Gourley et al., 2010). This suggests that PL is important in the acquisition of progressive ratio response requirements, but not the expression of behaviour once learnt.

In NHPs, aspiration lesions of dACC/24 typically bias monkeys to prefer low-cost/low-reward options over high-cost/high-reward options, although whether these effects are due to damaged grey matter or damage to underlying fibre tracts remains to be determined (Rudebeck et al., 2006, 2008; Walton and Mars, 2007). Electrophysiological studies have also shown that dACC/24 is one of the only regions of NHP PFC to be sensitive to effort cost (Kennerley et al., 2011; Wallis and Kennerley). Unfortunately, no studies have examined the effects of selective manipulations of NHP vmPFC on progressive ratio performance. The progressive ratio study presented in **Chapter 4** of this thesis represents the first of its kind – in which the effects of pharmacological manipulations of NHP vmPFC have been explored on motivational performance as assessed by this schedule of reinforcement.

In humans, questionnaires and interviews can be used to assess motivation by asking individuals about their drives and desires (Rømer Thomsen et al., 2015). However, as discussed in **1.5.2.3**, very few of the questionnaires in common usage assess motivational aspects of reward processing. Even the TEPS scale measures reward anticipation and does not directly assess reward motivation (Treadway and Zald, 2011). Nevertheless, isolated studies have endeavoured to more precisely assess motivational processes in questionnaires/interviews. In 2006, the Rhode Island Methods to Improve Diagnostic Assessment and Services project published work providing psychometric quantification of the DSM symptom criteria using structured interviews in over one-thousand patients (Zimmerman et al., 2006). The ‘diminished drive’ criterion (very similar to motivational

anhedonia) extracted from their interviews had the second highest odds-ratio for predicting a depression diagnosis – only sad mood was higher (McGlinchey et al., 2006).

In addition, instrumental tasks can also be used to assess willingness to work for reward in humans. An early example of this was used by Aharon and colleagues in 2001, to demonstrate a dissociation between motivational capacity and consummatory liking. In their paradigm, participants could 'work' to change the duration of viewing average or beautiful faces. Whilst heterosexual males rated beautiful female and male faces as equally (equal 'liking'), participants exerted more effort to keep female faces on the screen (differential 'wanting') (Aharon et al., 2001) showing that the constructs are behaviourally separable.

Subsequently, the Effort Expenditure for Rewards Task (EEfRT) was developed by Treadway *et al.* (Treadway et al., 2009) to more instrumentally quantify reward motivation in humans. EEfRT was designed based on fixed and progressive ratio studies in rodents (Aberman and Salamone, 1999; Salamone et al., 1994). In the EEfRT, decreased reward motivation is indicated by a reduced willingness to choose high-effort/high-reward options over low-effort/low-reward options. The sensitivity of the task was initially validated in healthy controls, where greater self-reported 'trait' anhedonia was associated with reduced willingness to choose high-effort/high-reward options. The EEfRT has also been used to assess motivational anhedonia in patient populations, where patient groups with either first-episode depression or remitted depression (together with 'at-risk' cohorts) show reduced effort expenditure for rewards (Treadway et al., 2012; Yang et al., 2014). This finding has also been reported in schizophrenic patients (Fervaha et al., 2013; Gold et al., 2013). Whilst these studies are informative, human participants are technically working for conditioned reinforcers (money) rather than primary rewards (such as food used in animal studies). Whether primary *vs.* secondary rewards are processed differently in the brain is unclear, but emerging evidence suggests that there may be differences (Sescousse et al., 2013b).

Neuroimaging studies in humans have given us several insights into the neurobiology of effort and motivation, and therefore the structures potentially disrupted in motivational anhedonia. Congruent with work in macaques, several imaging studies have found that activity within dACC/24 is associated with increasing effort requirements – interpreted as balancing effort expenditure with potential reward (McGuire and Botvinick, 2010; Prévost et al., 2010). As has been discussed, the vmPFC – particularly sgACC/25 and BA10 – has been implicated in subjective value encoding (Bartra et al., 2013). Effort is an important response cost that contributes to subjective reward value. Therefore, the question has been raised as to whether the vmPFC is involved in effort-related contributions to reward-based behaviour?

A recent study by Arulpragasam *et al.* dissociated neural correlates of effort- and reward-related contributions to decision-making by presenting subjects with two cues during fMRI: an effort-related cue and a reward-related cue, presented in either order (cue 1/2). Brain regions that were active when (i) only effort-related information was presented; (ii) only reward-related information was presented; or (iii) both sets of information had been presented could then be compared (Arulpragasam et al., 2018). Following cue presentation, subjects then made a choice between the effortful option and an alternative no-effort option. This study found that the anterior insula and dACC/24 encoded integrated cost-reward information at phase (iii). No region encoded effort alone (i), and both dACC/24 and vmPFC (sgACC/25, pgACC/32 and BA10) encoded reward-related subjective value information (ii). Arulpragasam investigated further by correlating neural signals with a 'subjective value prediction error': for instance, if subjects saw a high reward cue during cue 1, but then a high effort cue during cue 2, a negative subjective value prediction error would be expected following the presentation of cue 2. The dACC/24 and anterior insula (together with the caudate) reliably encoded subjective value prediction errors. Interestingly, in the vmPFC (BA10/25/32), if reward information was presented at cue 1, activity within this cluster closely tracked subjective expected value following cue 2 presentation. This activity was related to *expected* subjective rather than any objective information presented at the time of cue 1 because this cluster did not significantly respond to the reward magnitude of the presented option alone: similar vmPFC activity was observed in the case of low-value low-effort and high-value high-effort. This is the first human neuroimaging study to identify vmPFC as being engaged by expectations of reward in exchange for effort and suggests that PFC regions are computing integrated signals combining subjective value-related and effort-related information. Given that highly similar vmPFC subregions have been shown to be involved in depression and anhedonia (see **1.5.3.4.1**), this study could provide further insight into the mechanisms at play which underlie motivational anhedonia and associated impairments in decision-making.

1.5.3 Ventromedial prefrontal cortex in depression and anhedonia

Neuroimaging studies have consistently identified dysfunctional activity within the vmPFC associated with depression (Baxter et al., 1989; Biver et al., 1994; Drevets et al., 1992; Galynker et al., 1998; Greicius et al., 2007; Mayberg et al., 2005; Nofzinger et al., 2005). Building on primary evidence, several influential neurobiological models of depression directly implicate vmPFC dysfunction in its aetiology and/or pathogenesis (Palmer et al., 2015). Three particularly influential models include:

- The **limbic-cortical model** which emphasises the importance of interactions between dorsal prefrontal cortical areas, limbic and paralimbic (cingulate) structures;

- The **cortico-striatal model** which emphasises the importance cortico-striato-pallido-thalamic (CSPT) circuitry; and
- The **default mode network model** which emphasises the increased functional connectivity between sgACC/25 and the DMN, leading to maladaptive rumination and a lack of adaptive task-dependent modulation.

These models are not mutually exclusive, overlapping in terms of the neurobiological substrates being implicated and the consequences that dysfunction within these structures has on behaviour, physiology and cognition.

1.5.3.1 Limbic-cortical model

Building on earlier prototypes (Mayberg, 1994), in 1997 Helen Mayberg proposed a highly influential neurobiological account of depression, with the goal of linking impairments in cognition to sustained altered mood states characteristic of the disorder (Mayberg, 1997). This model was formulated based on several lines of evidence:

- **Blood flow changes during transient sadness in healthy subjects**. Induction of sadness in healthy controls results in a combination of limbic-cortical increases and decreases in metabolism (George et al., 1995; Pardo et al., 1993; Schneider et al., 1995). Specifically, both dorsal and ventral prefrontal regions are consistently implicated in the experience of 'normal' sadness – hypo-activity of dorsal regions and over-activity in ventral regions are amongst the most consistent findings (Mayberg, 1997). However, the precise nature of the change appears to be highly dependent on provocation method (Phan et al., 2002).
- **Resting state patterns of regional metabolism in patients with depression**. Depressed patients show altered patterns of resting-state regional metabolism including hypoactivity of a dorsal region corresponding to dlPFC/46 and dmPFC/9, and hyperactivity of caudal vmPFC corresponding to sgACC/25 (Mayberg, 1997). These loci are very similar to those identified in studies of transient normal sadness.
- **Changes in metabolism following successful antidepressant treatment**. The metabolic changes that characterise the depressed state appear to be sensitive to treatments. Whilst early work showed variable effects on dorsal frontal, dorsal cingulate and limbic regions associated with a variety of antidepressant treatment methodologies (Bench et al., 1995; Goodwin et al., 1993; Martinot et al., 1990; Nobler et al., 1994; Wu et al., 1992), differences in study design, cingulate location and analysis strategies renders direct comparison between these studies difficult.
  Using fluoxetine – an SSRI – in acutely depressed patients, Mayberg and colleagues assessed the responsivity of limbic-cortical regions to pharmacological intervention (Mayberg et al., 1999). Clinical responses were associated with metabolic changes in

dorsal prefrontal and vmPFC regions: the hypometabolic changes in dm/dlPFC were normalised, whereas the hypermetabolic sgACC/25 showed hypometabolism following successful treatment (activity below control levels). Non-responders with identical treatment showed persistently increased metabolism in sgACC/25. These divergent patterns suggest different adaptation patterns of brain regions to chronic 5HT modulation using SSRIs in responders *vs.* non-responders.

- **The value of pgACC/24,32 activity in predicting antidepressant response**. As mentioned above, there are consistent activity changes within dlPFC/dmPFC and sgACC/25 in depressed patients. However, patterns of activity in other brain regions are more inconsistent – in particular, there are contrasting reports of hypo- vs. hyper-activation of a perigenual vmPFC region corresponding to pgACC/24,32 (Drevets and Raichle, 1992; Drevets et al., 1992; Ebert and Ebmeier, 1996; Ebert et al., 1994). There could be several reasons for these differences, including variations in symptom clusters, medication status, illness severity and transient fluctuations in mood during imaging. Furthermore, volumetric changes in these structures can influence their activity as measured by functional neuroimaging – for instance, Drevets and colleagues initially reported hypoactivity in pgACC/32 associated with depression (Drevets et al., 1997). However, when corrected for reductions in volume, activity per unit volume seems to be *increased* (Drevets et al., 2008a).
  An alternative suggestion for this variability is proposed in the context of the limbic-cortical model. Mayberg and colleagues suggest that pre-treatment metabolic activity within this region might differentially predict the responsiveness of depressed patients to medication (Mayberg et al., 1997). Indeed, Mayberg *et al.* have shown that patients with high pre-treatment pgACC/24,32 activity show a robust response to antidepressants whereas patients with low pre-treatment activity typically failed to respond after six weeks. Interestingly, this same region has reciprocal connections with dmPFC/8,9 and ventral regions including sgACC/25. The integrity of this perigenual region may therefore be critical for normalising limbic-cortical dysfunction which accompanies the depressed state. Subsequent studies have supported the utility of pre-treatment activity in pgACC/24,32 in predicting treatment response (Boes et al., 2018; Klumpp et al., 2017).
- **Anatomical evidence** shows connectivity between vmPFC, dlPFC/dmPFC and brainstem structures involved in attention and cognition (Carmichael and Price, 1995; Pandya and Yeterian, 1996), establishing putative pathways through which limbic structures can modulate cognition (and vice-versa). Furthermore, these same regions and interconnected with subcortical and brainstem structures involved in visceral-autonomic regulation (see **1.3.1**). Therefore, dysfunction in these limbic-cortical

regions could impact upon several functions which are frequently disrupted in depression.

In the model, a *dorsal compartment* is proposed to be principally involved with the attentional and cognitive features of depression, including dm/dlPFC, dACC/24, parietal cortex and the dorsal striatum. A *ventral compartment*, consisting of limbic and paralimbic structures including sgACC/25, is proposed to mediate the vegetative and somatic aspects of depression. Isolated from both compartments is the rostral cingulate, corresponding to pgACC/24,32 where metabolism predicts treatment response in depressed individuals (Mayberg et al., 1997). PgACC/24,32 may serve as a regulator of the interaction between dorsal and ventral compartments, dysfunction in which can cause changes in remote brain regions to affect physiology, behaviour and subjective experience. See **FIGURE 1-27** for an overview of the limbic cortical model.

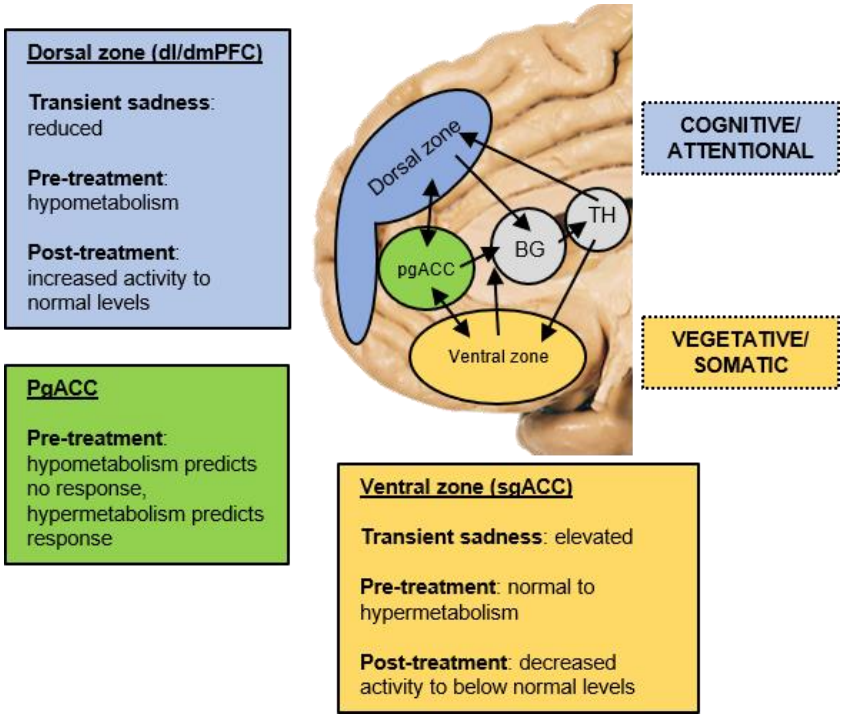


Figure 1-27 Limbic-cortical model. Adapted from Mayberg *et al*., 1997. The limbic-cortical model is an influential model of depression, implicating "*failure of the coordinated interactions of a distributed network of limbic cortical pathways.*" The regions implicated in this model are consistently identified in functional neuroimaging studies of normal sadness, baseline depressed patients and treatment recovery. Normal sadness and depression are associated with metabolic decreases in dorsal regions (dl/dmPFC and dACC/24; whose function is cognitive and attentional) and increases in more ventral regions (primarily sgACC/25; whose function is vegetative) – with successful treatment, these activity patterns are reversed. Metabolism in the perigenual cingulate (pgACC/24, 32) uniquely predicts antidepressant treatment response, and this region is thought to be critical for the adaptive changes associated with remission of depression.

At the core of the model is the hypothesis that depression is more than just dysfunction within a single compartment; rather, it is:

> *"…a failure of the coordinated interactions between the subcomponents of either compartment and between the two compartments."* (Mayberg, 1997)

Prior to the development of this model, functional studies of cognition had largely been carried out in isolation from functional studies of mood. However, the highly intercorrelated nature of the dorsal and ventral compartments during both pre- and post-treatment states suggests a fundamental link between brain regions typically associated with cognition and attention *vs.* brain regions associated with emotion and physiological regulation. In Mayberg's model, the negative influence of depressed mood on attention may therefore be attributable to functional connections between vmPFC and dm/dlPFC, rather than independent concurrent changes.

The limbic cortical model of depression has implications for treatment, specifically that treatments targeting components of either the dorsal or ventral compartments both represent viable options. Indeed, Mayberg and colleagues suggest that cognitive therapies such as CBT can be thought of as a top-down treatment strategy, augmenting the influence of dorsal neocortical regions on limbic/paralimbic pathways. Pharmacological therapies are typically thought of as acting in a bottom-up (or mixed) fashion: brainstem nuclei (such as the MRN/DRN) are major sites of antidepressant action and these project to structures in both compartments. An example of a bottom-up approach is leucotomy targeting vmPFC including sgACC/25, destroying putatively hyperactive limbic regions and potentially disinhibiting dm/dlPFC. Regardless of the initial causal perturbation, remodelling of limbic-neocortical circuitry would appear necessary to treat depression.

1.5.3.2 Cortico-striatal model

Abnormal CSPT circuitry has been proposed to explain, at least in part, clinical symptoms and cognitive deficits associated with depression. CSPT loops connect regions of the PFC – including vmPFC and dACC – with the basal ganglia and thalamus in a parallel but overlapping manner to support a multitude of behavioural and cognitive functions (Haber, 2016). Evidence for the importance of CSPT circuitry in mood disorders includes (i) structural and functional imaging studies showing evidence of alterations in CSPT components associated with depression (Furman et al., 2011; Marchand and Yurgelun-Todd, 2010; Rogers et al., 1998) and (ii) a higher prevalence of depression associated with neurodegenerative and vascular diseases with involvement of CSPT circuitry (Lauterbach et al., 1998; Marchand, 2010; Walterfang et al., 2011).

The ventral caudate and nucleus accumbens (forming, together with the olfactory tubercle, the ventral striatum) are arguably the most consistently implicated striatal subregions in depression. Patients with remitted depression show hyperactivation of the caudate and accumbens during negative picture viewing (Admon et al., 2015), and currently depressed patients show hypoactivation of the accumbens and ventral caudate during rewards (Pizzagalli et al., 2009; Smoski et al., 2009). Aberrant ventral striatal functional connectivity also predicts future risk for developing depression (Pan et al., 2017). Given the anatomical evidence that vmPFC subregions – particularly sgACC/25 – project strongly to the ventral striatum (Haber, 2016), vmPFC-ventral striatal limbic circuitry has been the focus of many studies examining CSPT changes associated with depression.

Several fMRI studies have revealed vmPFC-ventral striatal CSPT circuit changes in depressed subjects – including reduced activity within the vmPFC and ventral striatum during the anticipation of reward and during unexpected reward delivery (Segarra et al., 2016; Smoski et al., 2011). Keedwell *et al.* showed anhedonic symptoms (but not depression severity) were positively and negatively correlated with BOLD responses in vmPFC (BA10, 32)-amygdala and vmPFC-ventral striatum circuitry, respectively. Meta-analytic approaches have consistently identified volumetric abnormalities within limbic CSPT circuits: reduced volume in the prefrontal cortex – especially sgACC/25 and OFC – together with reduced volume in the ventral caudate and putamen (Bora et al., 2012; Koolschijn et al., 2009). Combining the results of multiple independent studies, a meta-analysis of functional resting-state network connectivity in depression has shown reduced connectivity between vmPFC and ventral striatum suggestive of blunted positive emotion and reward anticipation (Kaiser et al., 2015).

1.5.3.3 Default mode network model

Given the association between rumination in depression and the self-referential operations performed by the DMN (Raichle et al., 2001), it is perhaps no surprise that increased dominance of the DMN has emerged as a theory of depression. One of the regions most consistently implicated in the DMN is the rostral (r)vmPFC, corresponding to rostral BA10 and sometimes extending into frontopolar BA9 (Andrews-Hanna et al.). fMRI approaches have shown reliable increases in functional connectivity between the DMN (rvmPFC and posterior cingulate cortex, PCC) and caudal vmPFC, specifically sgACC/25, associated with depression (Hamilton et al., 2015). Thalamic involvement is also evident, with increased connectivity between sgACC/25, the mediodorsal thalamus (MDT) and DMN which has also been linked to higher levels of rumination (Berman et al., 2011; Zhu et al., 2012). Mutually propagating activation between sgACC and rvmPFC (via the thalamus) predicts high levels of rumination about depressive symptoms (Hamilton et al., 2011b).

What does this increased connectivity represent? Consider the function of sgACC/25. As discussed, elevated sgACC/25 activity has been seen during tonic and acute elevations in negative mood: at baseline in depression (Drevets et al., 1997; Mayberg et al., 1999, 2005); during negative affective stimulation in depression (Laxton et al., 2013); during transient sadness in healthy controls (Mayberg et al., 1999); and during neuroinflammatory challenge in healthy controls (Harrison et al., 2009). Caudal vmPFC subregions including sgACC/25 also have a physiological role in regulating parasympathetic tone (again, discussed previously). Related to its function in the context of the DMN, sgACC/25 has been implicated in behavioural withdrawal and energy conservation (Critchley et al., 2003; Matthews et al., 2005; Yang et al., 2009); given the role of these regions in negative affect, this suggests that the withdrawal is emotionally-laden, with a significant affective component.

These roles of sgACC/25 and the role of DMN in self-referential cognition and internalisation have led Hamilton and colleagues to propose that increased functional connectivity between DMN-sgACC/25 represents a neural correlate of increased depressive rumination. In depression, the function of the DMN – biasing towards self-referential thinking processes – and the sgACC/25 – supporting negatively affectively-laden behavioural withdrawal – are linked, resulting in pathological rumination: self-focused, negatively valenced and withdrawn thinking processes (**FIGURE 1-28**). This union underlies maladaptive thought patterns. Interestingly, this model also proposes an explanation for the increased functional connectivity between MD thalamus and DMN in depressed patients. SgACC/25 does not directly project to nodes of the DMN (except for subregions of the rvmPFC) (Johansen-Berg et al., 2008), but does project to MD thalamus (which itself projects to DMN components) suggesting that the increased correlation of activity between sgACC/25 and DMN is (at least in part) mediated by projections through MD thalamus.

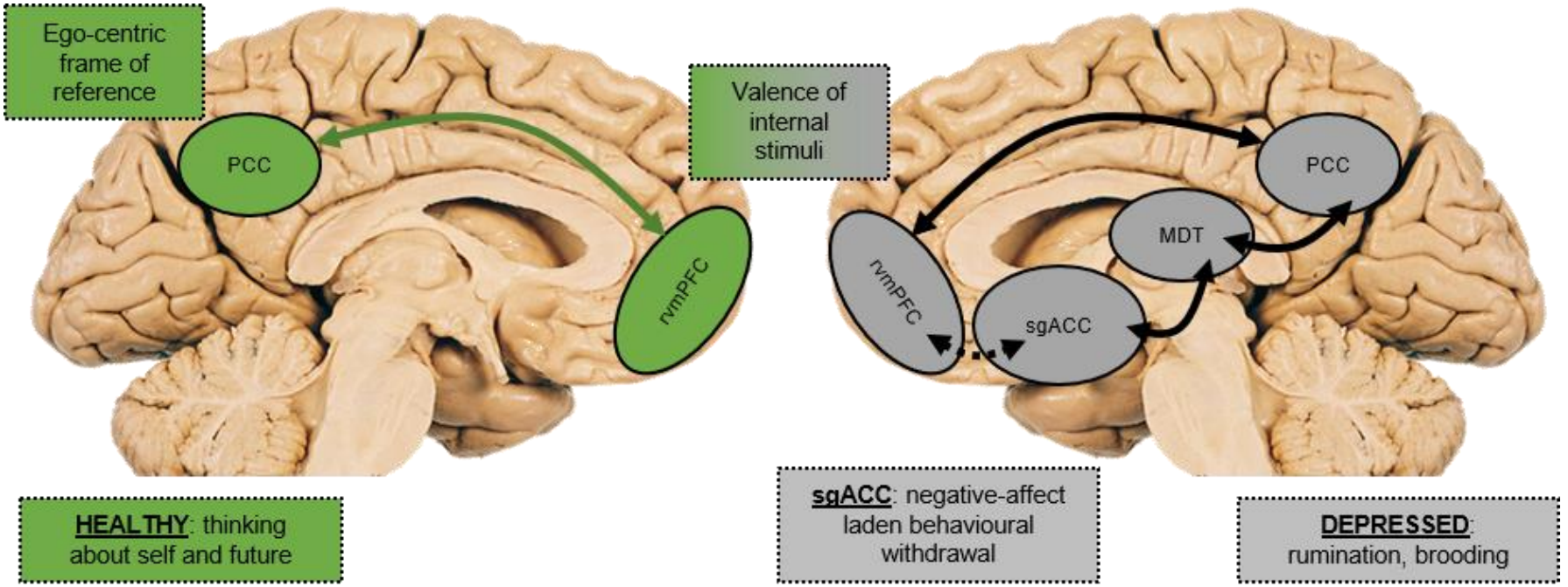


Figure 1-28 Default mode network (DMN) model. Adapted from Hamilton *et al*., 2015. In the healthy state, the DMN is responsible for self-referential thinking. The rostral vmPFC (rvmPFC) and posterior cingulate cortex (PCC) are most consistently implicated in the DMN. Hamilton and colleagues suggest that in depression, the internalising function of the DMN and the negative affect-laden behavioural withdrawal functions of sgACC/25 are abnormally linked. Observations of increased functional connectivity between the mediodorsal thalamus (MDT) and DMN are also explained by this model: sgACC/25 does not directly project to components of the DMN (except for portions of rvmPFC) but does project to the MD thalamus (MDT), suggesting that increases in correlated activity between sgACC/25 and DMN are mediated by projections through MD thalamus.

1.5.3.4 Clinical implications of vmPFC dysfunction in depression

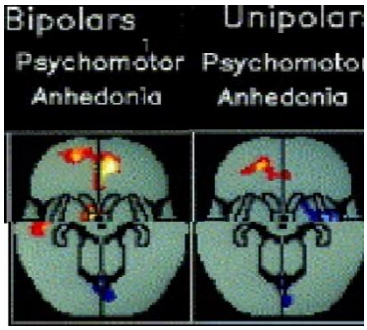
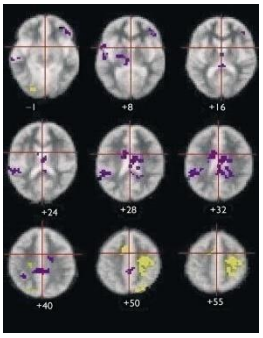
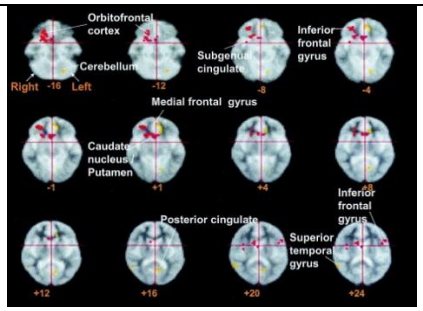
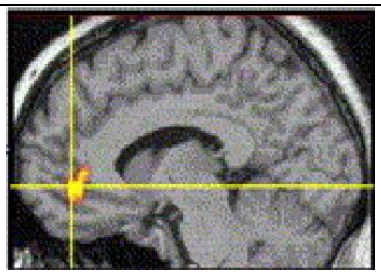
An understanding of vmPFC function has clinical significance regarding the diagnosis and treatment of depression for several reasons:

- **Which symptoms of depression are *specifically* linked to vmPFC dysfunction?** Given that depression is a heterogeneous condition, it is critical to understand vmPFC dysfunction in the context of specific symptoms, rather than in a nebulous clinical construct. Indeed, this is the impetus behind the Research Domain Criteria (RDoC) approach, spearheaded by the USA's National Institute of Mental Health. The RDoC approach explores basic 'dimensions of functioning,' spanning the full range of human behaviour from normal to abnormal. For example, physiological systems involved in reward processing, when dysfunctional, manifest as anhedonia – whereas physiological systems involved in punishment, when dysfunctional, could manifest as anxiety/low-mood. As a syndrome, depression can manifest as varying degrees of dysfunction within separable physiological systems. It is important to understand the neurobiology and neuropathology underlying these specific domains of functioning and their disruption, to better account for individual variability in symptoms.
- **Is vmPFC activity a biomarker of treatment response?** vmPFC activity in baseline or task-related settings may reflect a neural biomarker of a patient's sensitivity to different treatment modalities. If this were the case, it would be advantageous for clinicians when identifying optimum treatments.
- **Could vmPFC represent a viable treatment target?** Given its extensive involvement in depressive symptoms, the vmPFC is a candidate brain region for targeted interventions in the treatment of depression – including DBS (Mayberg et al., 2005).

1.5.3.4.1 vmPFC linked to specific symptoms of depression: low mood and anhedonia

The two core symptoms of depression are enhanced negative mood and anhedonia. As outlined in **1.5.3.1**, extensive evidence implicates vmPFC in enhanced negative mood: transient sadness induction studies in healthy controls; blood flow changes in the resting state of depressed patients; and changes associated with successful depression treatment all point to a role of vmPFC subregions. Note that the latter two lines of evidence link altered activity in vmPFC to the disorder, and not the symptom per-se.

Comparatively few studies have assessed the role of vmPFC subregions in anhedonia. Those that have implicate both state anhedonia in depressed patients and trait anhedonia in healthy controls to over-activity in a rostral region of vmPFC corresponding to BA10 and pgACC/32 (**TABLE 1-9**).

Reference	Image	Description
	Human vmPFC in anhedonia	
(Dunn et al., 2002)	 BA10, 24, 25, 32	^{18}F-FDG PET, continuous performance task: the psychomotor anhedonia cluster of the Beck Depression Inventory correlates with higher activity in anterior cingulate and vmPFC regions in both unipolar and bipolar depressed patients.
(Mitterschiffthaler et al., 2003)	 BA10, 24 (purple = increased activity)	fMRI, picture viewing: Depressed patients show increased activation of BA10 and dACC/24 during the presentation of positive stimuli.
(Kumari et al., 2003)	 BA10, 24, 25 (red = increased activity)	fMRI, picture viewing: Depressed patients show increased response in BA10 and sgACC/24,25 associated with reduced positive emotion whilst viewing positive pictures.
(Keedwell et al., 2005)	 BA10, 24, 32	fMRI, picture viewing: Increased response of rostral vmPFC region to happy stimuli correlated with anhedonia severity and negatively correlated with happy mood ratings.

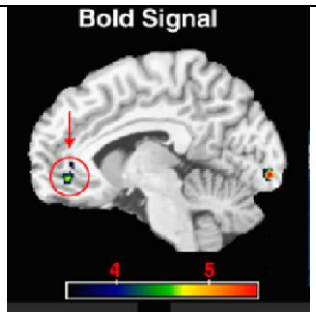
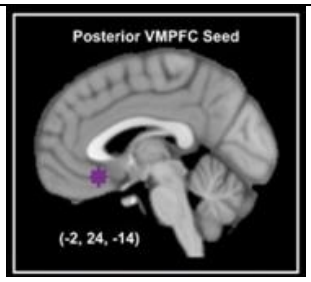
(Harvey et al., 2007)	 BA10, 32	fMRI, picture viewing: NB/ trait anhedonia assessed in healthy controls. pgACC/32 activity positively correlated with trait anhedonia during processing of pleasant information (not during negative information).
(Young et al., 2016)	 BA10, 25	fMRI, resting-state and task-based: this study used a caudal vmPFC subregion (BA10, 25; identified based on activation likelihood meta-analysis of studies implicating vmPFC in depression) as a seed region. Connectivity of this region to reward-related structures is negatively correlated with anhedonia (but not general distress) during positive music listening.

Table 1-9 Neuroimaging studies showing vmPFC activity associated with anhedonia. Activity within a rostral vmPFC region corresponding to pgACC/32 and BA10 is associated with increased anhedonia in healthy controls (trait anhedonia) and in depressed patients (state anhedonia). Some studies also show changes in function/connectivity within a caudal, subgenual region associated with anhedonia (Dunn et al*.,* 2002; Young et al., 2016).

Keedwell *et al.* examined the neural responses to happy and sad autobiographical memory stimuli to determine whether anhedonia severity (assessed using the FCPS) is positively correlated with vmPFC activity and negatively correlated with ventral striatal activity during the presentation of positive (and not negative) stimuli (Keedwell et al., 2005). Their study found that a large area of vmPFC (predominantly pgACC/32 and BA10) showed greater activation to happy stimuli correlated with anhedonia severity; by contrast, large areas of the ventral striatum showed reduced activation to happy stimuli correlated with anhedonia severity. No correlation was found between anhedonia severity and responses in reward areas to sad stimuli, suggesting that these patterns of activity change are strictly related to appetitive stimuli. The conclusions that can be drawn from this study are somewhat limited as all but one of the participants were taking antidepressants.

Further insight into the role of the rostral vmPFC in anhedonia comes from neuroimaging studies in healthy controls assessing trait anhedonia (Harvey et al., 2007). Trait anhedonia is

an enduring personality trait that varies within the population (Blanchard et al., 2001), and can be considered a vulnerability factor for neuropsychiatric illness (Hasler et al., 2004; Pizzagalli et al., 2005); therefore, structural changes apparent in individuals with high trait anhedonia could provide insight into changes occurring early-on in the disease course. An additional advantage conferred by studying trait anhedonia in healthy controls *vs.* state anhedonia in depressed patients relates to disease-associated confounds. In depressed cohorts, it is difficult to disentangle changes in brain activity related to anhedonia, changes related to other symptoms, and changes related to stable personality traits *vs.* acute changes in clinical state. Harvey *et al.* assessed trait anhedonia using the CPAS and related this to brain activity during the presentation of positive and negative affective picture stimuli. In addition to demonstrating a negative correlation between anterior caudate volume and trait anhedonia, a significant positive correlation was identified between BOLD signal in vmPFC (BA10, 14 and 32) and trait anhedonia. Note that in both Harvey *et al.* and Keedwell *et al.*, the measure of anhedonia is a questionnaire, and it remains unclear whether self-report anhedonia is more closely associated with deficits in hedonic processing, anticipatory processing or motivational function.

Elevated activity within the vmPFC associated with state and trait anhedonia could be interpreted in two ways. First, in depressed patients, prefrontal areas are 'over-inhibiting' subcortical emotion-generating regions during the presentation of positive stimuli, suggesting a primary problem in the vmPFC. Second, the increase in activity is compensatory, directing attention to positive stimuli to improve mood, suggesting a primary problem in other structures. Given that several subcortical structures such as the striatum and amygdala show decreased volumes associated in MDD (Beyer and Krishnan, 2002), this might suggest a primary subcortical cause with prefrontal compensation. However, further work is needed to clarify this. Indeed, work presented in **Chapter 4** of this thesis suggests that over-activity within sgACC/25 is sufficient to cause the physiological and behavioural correlates of anticipatory and motivational anhedonia.

More recently, Young and colleagues directly addressed the role of *caudal* vmPFC (corresponding to sgACC/25) in anhedonia, attempting to address the discrepancy between the large body of work implicating sgACC/25 in depression, and the dearth of work implicating this region in anhedonia (Young et al., 2016). Anatomical evidence showing connectivity between sgACC/25 and components of the reward system – such as the nucleus accumbens (Ongür and Price, 2000) – would suggest that dysfunction within this prefrontal region would have an impact upon reward processing. Young and colleagues identified an sgACC/25 ROI based on a review of vmPFC subregions implicated in mood disorders (see **TABLE 1-9**). In patients with depression, connectivity of this region to key

emotion and reward-related structures (including ventral striatum) was negatively correlated with anhedonia (but not general distress) during positive music listening. Resting state sgACC/25 connectivity did not distinguish anhedonia from general distress in either health controls or depressed patients. This demonstrates that anhedonia is associated with task-dependent engagement of sgACC/25 connectivity when encountering pleasant stimuli, but not a static difference in intrinsic resting-state function.

1.5.3.4.2 vmPFC as a biomarker for treatment response

Activity and connectivity of the vmPFC has shown promise as a predictor of response to multiple different types of treatments.

- **Pharmacotherapy**. Pre-treatment resting-state functional connectivity between posterior vmPFC (sgACC/25 and BA10) and dACC correlates with successful treatment outcome (Kozel et al., 2011).
- **CBT**. Pre-treatment activity in rostral vmPFC (BA10) has been positively associated with responses to CBT (Ritchey et al., 2011), whereas activity in caudal vmPFC (caudal BA10) has been negatively associated with CBT outcomes (Siegle et al., 2006).
- **rTMS**. The efficacy of rTMS to dlPFC is related to higher pre-treatment resting-state functional connectivity between vmPFC and dmPFC (Salomons et al., 2014), and between vmPFC, striatum, VTA and dmPFC (Downar et al., 2014b).
- **ECT**. Redlich and colleagues identified a positive association between pre-treatment sgACC/25 volume and individual responses to ECT (Redlich et al., 2016).
- **DBS**. Responders to DBS of white matter underlying sgACC/25 show stronger connectivity between sgACC/25, dACC/24 and ventral striatum (Riva-Posse et al., 2014).

Broadly speaking, it seems the effects of various treatment modalities can be predicted by neuroimaging correlates of vmPFC function. Consistent with these findings, changes in vmPFC BOLD signal have been identified pre-treatment to post-treatment. A meta-analysis has shown increased anterior vmPFC activity (BA10) in response to positive emotions following successful pharmacological treatment (Ma, 2015). Reduced activity in a region of posterior vmPFC including sgACC/25 has been observed following successful pharmacological, surgical and rTMS treatment of depression (Hiser and Koenigs, 2018). These data would suggest separable roles for rostral and caudal zones of vmPFC in the response to depression treatments.

1.5.3.4.3 vmPFC as a target for treatment

The apparent pivotal role of vmPFC dysfunction in depression has meant it has become a popular target for neurosurgical interventions. The first of such interventions were surgical ablations of vmPFC and its efferent pathways to treat refractory depression, including subcaudate tractotomy (Bridges et al., 1994) and leucotomy (Sachdev and Sachdev, 2005). Although reasonably efficacious, these strategies are associated with gross damage to relatively large regions of cortex/white matter, and have several side-effects (Catani et al., 2013).

One of the most promising surgical interventions was developed in 2005, involving targeted DBS of grey matter (and adjacent white matter) in sgACC/25 (Mayberg et al., 2005). DBS involves stereotactic implantation of electrodes into a specific brain region. These electrodes are connected to a subcutaneous generator which provides power and controls stimulation (typically continuous). DBS is relatively well-tolerated and the most common adverse effects are associated with the neurosurgery rather than long-term issues with the implant itself (Delaloye and Holtzheimer, 2014).

The initial proof-of-concept study implanted DBS electrodes into six patients with treatment resistant depression. Following six months of chronic grey/white matter sgACC/25 DBS, four of these patients were in remission/near remission (Mayberg et al., 2005). An expanded study including 20 patients showed a 60% one-year response rate with a remission rate of 50% (Lozano et al., 2008). Encouragingly, symptoms of depression associated with enhanced negative affect, reduced positive affect and disrupted cognition all improved. Subsequent studies of sgACC/25 DBS have shown remission rates associated with chronic stimulation ranging from 33% to 58% (Delaloye and Holtzheimer, 2014). In a recent double-blind sham-controlled trial, Puigdemont and colleagues reported reversal of symptoms in five treatment resistant patients, with long-term high frequency stimulation associated with optimal antidepressant response (Puigdemont et al., 2015). Blinded discontinuation of DBS is associated with a resurgence of depressive symptoms which ameliorate when stimulation is restarted (Holtzheimer et al., 2012).

As more and more studies investigate the efficacy of sgACC/25 DBS, optimal stimulation parameters are also being elucidated. Longer pulse durations influence pathways further from sgACC/25, with suggestions that activation of the sgACC/25-accumbens network could contribute to the antidepressant response (Johansen-Berg et al., 2008). Furthermore, the optimal electrode placement does not appear to be in grey matter of sgACC/25 – rather, it lies in adjacent white matter (Lozano et al., 2012). It appears that the beneficial effects of sgACC/25 DBS are associated with a direct impact on multiple fibre bundles passing through sgACC/25. These include:

- **Bilateral forceps minor of the anterior corpus callosum** connecting right and left mPFC;
- **Bilateral cingulum bundles** connecting ipsilateral sgACC/25 to pgACC, dACC and mid-cingulate cortex;
- **Bilateral medial branch of the uncinate fasciculus** connecting sgACC/25 to rostral vmPFC anteriorly, and to accumbens, thalamus and other subcortical regions posteriorly; and
- **Frontostriatal fibres** connecting sgACC/25 to ventral caudate and nucleus accumbens.

Tractography in individual patients may facilitate optimal placement of electrodes within white matter, given that these critical neuronal tracts appear to be necessary for the antidepressant response. Riva-Posse and colleagues have shown that electrode placements can be successfully selected based on individual tractography maps (Riva-Posse et al., 2014). After one year of stimulation, nine of eleven patients were responders and six were in remission. This highlights the potential utility of connectomic approaches to guide future sgACC/25 DBS surgical targeting.

1.5.4 Anxiety disorders

1.5.4.1 Defining Anxiety

In defining anxiety, it is important to distinguish the construct from fear. Fear and anxiety are overlapping states centred on threat. In some ways, these two constructs are similar: both involve negative subjective feelings, and both involve bodily manifestations. However, in several ways they are distinct (Öhman, 2008):

- **Subjective experience**: Fear has been described as a sense of *impending disaster* eliciting a *defensive reaction* (fight or flight) whereas anxiety is associated with an *ineffable and unpleasant feeling of foreboding*.
- **Identifiable eliciting stimulus**: Fear has an identifiable *eliciting stimulus* (with a definite location in space and time) whereas in anxiety the nature and location of the threat is obscure (except e.g. phobias).
- **Temporal relationship to potentially aversive outcome**: Fear is often *peri-stimulus* whereas anxiety is often *pre-stimulus* (an anticipatory response to threatening stimuli).

Anxiety is an adaptive response, but if unregulated or inappropriately regulated, it becomes biologically and socially maladaptive, impairing daily function. The transition from an adaptive to maladaptive response represents the transition from physiological to pathological anxiety – an anxiety disorder.

1.5.4.2 Classifying Anxiety Disorders

Anxiety disorders are a heterogeneous group of conditions and the classification of anxiety disorders is subject to continuous flux. From DSM-IV to DSM-V, the category of *Anxiety Disorders* split into three separate categories (American Psychiatric Association, 2013):

1. **Anxiety Disorders** including separation anxiety disorder, selective mutism, specific phobia, social phobia, panic disorder, agoraphobia and generalised anxiety disorder (GAD).
2. **Obsessive-Compulsive Disorders** including OCD, body dysmorphic disorder, hoarding disorder, trichotillomania and excoriation disorder.
3. **Trauma and Stressor-Related Disorders** including reactive attachment disorder, disinhibited social engagement disorder, PTSD, acute stress disorder and adjustment disorder.

The prototypical anxiety disorder is Generalised Anxiety Disorder (GAD). GAD is defined as at least 6 months of excessive worry about everyday issues in a manner that is disproportionate to any inherent risk, causing significant distress or impairment. According to DSM-V, at least three symptoms out of a possible six are needed to make a diagnosis (American Psychiatric Association, 2013): restlessness or nervousness, easy fatigability, poor concentration, irritability, muscle tension or sleep disturbance. Other neuro-vegetative complaints are also common such as sweating, palpitations, dizziness and gastric discomfort. The diagnosis is one of exclusion, because clinicians need to rule out potential causes (including other medical conditions, medications or substances) before a diagnosis of GAD can be proposed.

1.5.4.3 Epidemiology of Anxiety Disorders

Collectively, anxiety disorders are the most prevalent mental disorders, associated with large societal burden (Bandelow et al., 2017). Approximately one-third of the population is affected by an anxiety disorder at some point in their lifetime. Female:male ratios for prevalence rates are somewhat variable, although studies consistently show that anxiety disorders are more prevalent in women (Lieb et al., 2005). Prospective studies suggest that anxiety disorders are chronic, starting in childhood, adolescence or early adulthood, reaching peak prevalence in middle age and then becoming less common in elderly cohorts (Lenze and Wetherell, 2011).

1.5.4.4 Aetiology and Pathophysiology of Generalised Anxiety Disorder

1.5.4.4.1 Genetic accounts

Genetic studies show a moderate a degree of heritability, with genetic linkage accounting for approximately one-third of the variability in trait levels of anxiety in children (Albano et al.,

2003). Symptoms of anxiety disorders show increased correlation amongst monozygotic twins compared to dizygotic twins (Eley et al., 2015). A meta-analysis of GWAS studies in GAD has found that different studies identify different genome-wide significant regions, suggesting that there are small contributions of multiple individual genes, typical of a polygenic pattern of inheritance (Otowa et al., 2016).

1.5.4.4.2 Behavioural accounts

A classical learning theory approach posits that patients develop an anxiety response to a situation when they have been previously punished in those same situations, resulting in the formation of an aversive conditioned emotional response. Avoidance responses are triggered by negative reinforcement, and this instrumental behaviour prevents extinction of the conditioned emotional association, maintaining the maladaptive anxiety. This is formally proposed in Mowrer's two factor theory of fear and anxiety (Mowrer, 1960): classically conditioned acquisition of fear is followed by operantly conditioned avoidance of fear cues leading to fear maintenance due to a lack of unreinforced exposure that would normally cause extinction of the conditioned response. Whilst intuitive, Mowrer's account does not explicitly differentiate between physiological, adaptive anxiety and pathological anxiety interfering with daily function. Furthermore, it does not explain why certain individuals are more likely to develop maladaptive patterns of behaviour.

1.5.4.4.3 Cognitive accounts

As is the case in depression, it is appreciated that cognitive biases contribute to pathological phenotype of anxiety. Not only can cognitive factors influence behavioural avoidance, but patients can exhibit a cognitive (imagined) avoidance. This typically involves shifts in attention away from the negative stimulus. Borkovec posited that cognitive avoidance may (i) contribute to the initial development of anxiety (excessive focus on potential threat); (ii) facilitate the maintenance of anxiety (through the redirection of attention); and (iii) mitigate the effect of behavioural therapies such as flooding (Borkovec, 1985). Indeed, according to the DSM-V, the central defining characteristic of GAD is a cognitive process, 'worry,' which is closely linked to rumination and self-directed attentional processes (American Psychiatric Association, 2013).

1.5.4.4.4 Stress and dysfunction within the HPA axis

Single major negative life events increase the risk of developing GAD threefold (Blazer et al., 1987). In the year preceding diagnosis, male patients tend to report financial and occupational stressors as events precipitating pathological anxiety, whereas female patients tend to report parenting stress, financial stress and interpersonal problems as precipitant (see *BMJ Best Practice: Generalised anxiety disorder*, https://bestpractice.bmj.com/topics/en-gb/120). A history of early childhood separation is also more common in GAD populations

(Taher et al., 2015). In addition to these major negative life events, patients with GAD report significantly more minor life events compared to non-anxious controls, and patients perceive these events as being significantly more stressful than non-anxious controls (Brantley et al., 1999).

Given its role in the stress response, the HPA axis would be a major candidate for a physiological system in which disruption leads to anxiety disorders. However, despite this rationale, plasma cortisol and CRH levels are normal in patients with GAD (*c.f.* patients with depression) (Catalán et al., 1998; Kelly and Cooper, 1998). Other studies using the dexamethasone suppression test as a probe of HPA axis function have reported a non-suppression rate of only 30% (compared to 50% in depressed patients) suggestive of potential dysfunction in cortisol regulation in a subset of patients with GAD (but certainly not all patients) (Tiller et al., 1988).

1.5.4.4.5 Neurobiological changes

The diversity of neurobiological structures involved in 'normal' anxiety responses (**FIGURE 1-29**) is reflected in the myriad of neuroanatomical changes that have been implicated in the aetiology and pathophysiology of anxiety disorders, including changes in the hippocampus (Yamasue et al., 2008), amygdala (De Bellis et al., 2000) and vmPFC (these are discussed in more detail below, see **1.5.5**).

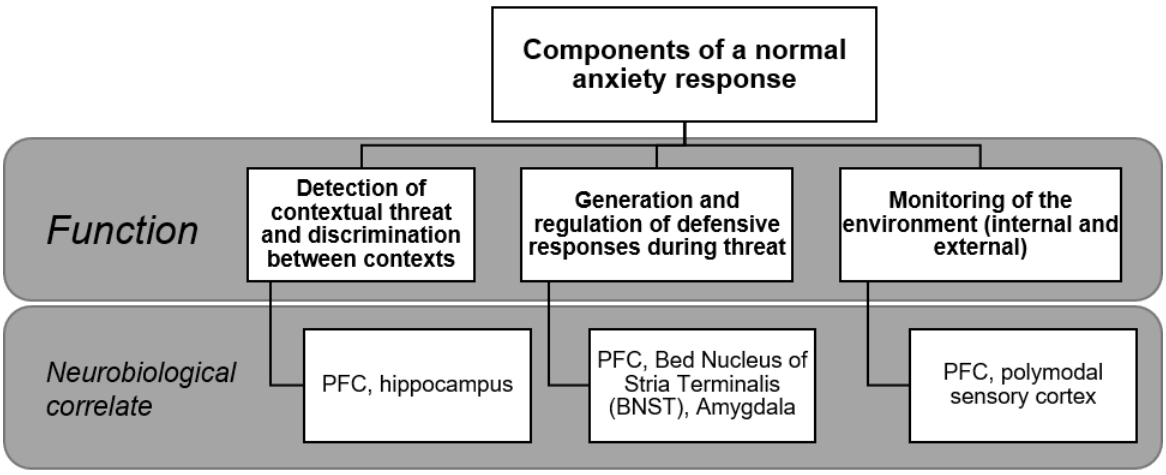


Figure 1-29 Components of a normal anxiety response and their neurobiological correlates. Anxiety responses involve at least three components: (i) detection of contextual threat and discrimination between contexts; (ii) generation and regulation of defensive responses and (iii) continual monitoring of the environment. The hippocampus is critical in contextual monitoring and discrimination, as revealed by the impairments in contextual conditioning associated with lesions of the ventral hippocampus in rodents (Phillips and LeDoux, 1992). The Bed Nucleus of the Stria Terminalis (BNST) has an established role in sustained fear/anxiety, where it mediates the

autonomic, endocrine and behavioural responses during situations of threat (Goode and Maren, 2017; Lebow and Chen, 2016). CRH-containing neurons in the BNST output to the amygdala, which in turn can activate the HPA axis (Davis et al., 2010). Unimodal and polymodal sensory cortex are important in continual monitoring of the environment. Various subregions of the PFC have been implicated in all of these functions, together with an integrative function in coordinating physiological, behavioural and cognitive responses.

1.5.4.4.6 Dysfunction within neurotransmitter systems

Given the function of benzodiazepines – first line anxiolytic drugs – in enhancing GABAergic transmission, this neurotransmitter has been extensively implicated in the pathophysiology of anxiety disorders. Reduced GABA receptor levels; changes in levels of endogenous allosteric GABA receptor modulators; and changes in subunit composition of GABA receptor levels have all been proposed as mechanisms by which neuronal inhibition is reduced in pathological anxiety (Nuss, 2015). GABAergic tone in the amygdala seems to be particularly important – direct infusions of GABA agonists into the amygdala reduce measures of fear and anxiety in preclinical models (Barbalho et al., 2009), and benzodiazepine administration specifically attenuates amygdala activation to fearful stimuli in humans (Del-Ben et al., 2012). GABAergic interneurons of the amygdala are found in the intercalated cell masses, which receive the main glutamatergic input from IL and are well-placed to gate information transfer within the amygdala.

Anxiety disorders also involve alterations in several of the monoamines, including 5HT and NA. Until relatively recently, the precise contribution of 5HT to anxiety was controversial. For example, increased 5HT levels appear to be ‘anxiogenic’ in response-conflict models, whereas escape models suggest 5HT is ‘anxiolytic’ (Graeff, 2002). To explain these findings, it has been suggested that inhibitory avoidance (exhibited during response-conflict procedures) *vs.* escape represents anxiety *vs.* panic, and that 5HT had different roles in these emotions. Deakin and Graeff suggest that *avoidance* is enhanced by 5HT release in vmPFC and amygdala, whereas *escape* is inhibited by 5HT release in the dorsal PAG (Deakin and Graeff, 1991; Graeff, 1991) (**FIGURE 1-30**). In terms of translation to the clinical state, inhibitory avoidance behaviours are more relevant to GAD, whereas escape behaviours are related to panic disorder. Given that MRS techniques cannot measure levels of 5HT, new techniques are needed to enable targeted measurement of 5HT levels in vmPFC/dorsal PAG, to compare between healthy controls and GAD patients.

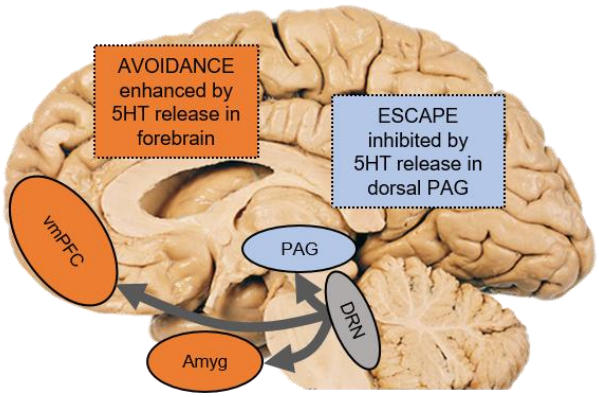


Figure 1-30 Deakin-Graeff model of 5HT in anxiety and panic. Until the late 20th/early 21st century, the role of 5HT in fear and anxiety was highly controversial. Whilst manipulations targeting the 5HT system suggest an anxiogenic role of 5HT in conflict models, escape models suggested an anxiolytic role. To resolve this apparent disparity, Deakin and Graeff suggest that *avoidance* behaviours represent anxiety, whereas *escape* behaviours represent panic – and that 5HT has different roles in these functions. Accumulating evidence points specifically to 5HT in the forebrain (vmPFC/amygdala) promoting avoidance, and 5HT in the dorsal PAG inhibiting escape.

The elevated T-maze has been developed as a paradigm to simultaneously assess of both escape and avoidance behaviours in rodents, and therefore provide evidence for this hypothesis. The T-maze consists of three arms – two open arms and one enclosed arm. Following familiarisation with the maze over several multiple trials, rodents exhibit inhibitory avoidance when placed in the enclosed arm, refraining from exploring the maze owing to the unpleasant nature of the open arms. By contrast, when placed in an open arm, rodents will perform an escape response to reach the safe closed arm (Zangrossi and Graeff, 2014). Selective lesions of the DRN using the 5HT toxin 5,7-dihydroxytryptamine (5,7DHT) impairs avoidance when placed in a closed arm (anxiolytic) but facilitates escape in the open arm (panicogenic), consistent with the Deakin-Graeff model (Sena et al., 2003). Chronic administration of drugs used to treat panic disorder have been shown to enhance the inhibitory effects of 5HT in the dorsal PAG on escape behaviour via receptor sensitisation (see (Zanoveli et al., 2005) using imipramine) and enhanced 5HT efflux (Zanoveli et al., 2010) thereby having a dual action to increase 5HT levels in dorsal PAG. The pattern of changes induced by 5HT manipulations of the adjacent MRN are different: 5,7DHT lesions to the MRN also have anxiolytic effects on inhibitory avoidance but do not affect escape behaviour (Andrade et al., 2004).

Enhancement of $5HT_{2C}$-mediated neurotransmission in the amygdala is thought to underlie the acute anxiogenic effects of TCAs/SSRIs seen in the first few days of treatment (Sinclair et al., 2009) – indeed, intra-BLA injections of the $5HT_{2C}$ agonist MK212 elevates inhibitory

avoidance but does not affect escape (Vicente and Zangrossi, 2012). The anxiogenic effects of the antidepressant imipramine are completely blocked by intra-BLA administration of the $5HT_{2C}$ antagonist SB-242084 (Vicente and Zangrossi, 2012). The anxiolysis associated with chronic antidepressant treatment appears to be related to *desensitization* of $5HT_{2C}$ receptors in the BLA, causing an anxiolytic (rather than anxiogenic) effect (Zangrossi and Graeff, 2014).

The role of catecholaminergic neurotransmission in stress has also led to its investigation as a candidate for dysfunction in anxiety disorders. Some studies suggest over-activity of NA locus coeruleus neurons is associated with pathological anxiety, although data are inconsistent (Nutt, 2001). A recent meta-analysis has shown that treatment with SNRIs such as venlafaxine and NA reuptake transporter inhibitors such as reboxetine and atomoxetine are efficacious, and associated with *reduced* incidence of panic disorder and reduce phobic symptoms (Montoya et al., 2016). Therefore, contrary to the prediction made by the enhanced NA transmission hypothesis, drugs which increase NA levels in synaptic clefts appear to ameliorate anxiety symptoms. However, further work is needed to comprehensively delineate the contributions of noradrenergic transmission to anxiety.

1.5.4.5 Treatment of Anxiety Disorders

Treatment of anxiety disorders is aimed at improving symptoms and reducing/eliminating disability. Pharmacotherapy is considered first line, but CBT and cognitive therapy are considered equal first-line treatment options, especially in patients who do not wish to take drugs.

Amongst options for pharmacotherapy, first line treatments are predominantly SSRIs (citalopram, paroxetine and sertraline) (Gale and Oakley-Browne, 2005), SNRIs (duloxetine, venlafaxine) (Nicolini et al., 2009) or pregabalin (Bandelow et al., 2012). SSRIs have shown efficacy in treating GAD in children, adolescents and elderly patients (Ipser et al., 2009; Schuurmans et al., 2009) and in preventing relapse (Allgulander et al., 2006). In patients with co-morbid depression, SNRIs have been shown to be particularly effective (Mancini et al., 2010). Long-acting benzodiazepines such as clonazepam can be used at the start of SSRI/SNRI therapy to attenuate some of the side effects during the delay in onset of action (approximately 4 weeks) (Bandelow et al., 2012) provided patients do not have a history of substance abuse.

1.5.4.6 Animal Models and Tests of Anxiety-Like Behaviour

Attesting to either their versatility, imprecision or perhaps both, animal models of anxiety disorders are very similar to those of depression, including as chronic stress (Campos et al., 2013). The correspondence between these models may also reflect similar underlying

neurobiological bases, or even that the disorders are so inter-related that they are inseparable at the resolution of preclinical models. As has been mentioned, depression and anxiety disorders are highly comorbid (Lamers et al., 2011).

Several tests of innate (sometimes termed 'unconditioned') anxiety have been developed for use in rodents, such as the elevated T-maze, elevated plus maze, open field test and light-dark box (Kumar et al., 2013). These assays exploit the conflict between an animal's innate drive to explore, and the inhibition associated with a novel, mildly aversive environment (either brightly lit or exposed) (McCormick and Green, 2013). For instance, in the elevated plus maze, the animal is placed in the centre of an elevated platform with two open arms and two enclosed arms (Pellow et al., 1985). The open arms combine elements of unfamiliarity, openness and elevation. In this model, there is a drive to explore the open arms but a conflicting aversion to the elevated, open spaces. Indeed, the elevated plus maze has been referred to as an *unconditioned spontaneous behavioural conflict model* (Bourin et al.). The proportion of time spent in the open *vs.* closed arms is computed as a measure of the animal's state anxiety.

Tests such as the elevated plus maze are practically straight-forward to carry out. Construct validity of this test has been demonstrated, as anxiogenic drugs reduce the proportion of time spent in open arms whereas anxiolytic drugs increase this measure (Pellow et al., 1985). The main measure used in the elevated plus maze shows face validity – but additionally, levels of freezing/immobility behaviours and defaecation are higher in the open arms (Pellow et al., 1985) showing that animals exhibit a repertoire of anxiety-like behaviours on this task. Attesting to the predictive validity of this test, it has been frequently used to pre-screen new medications for anxiety disorders (Walf and Frye, 2007). Despite these advantages, it cannot be known whether these assays are measuring a sense of 'ineffable foreboding' or 'worry'.

1.5.5 Ventromedial prefrontal cortex in anxiety disorders

The extensive involvement of vmPFC in depression implicates it in anxiety by virtue of the significant overlap in incidence and symptom criteria between the two disorders (Ressler and Mayberg, 2007). Given that multiple lines of evidence implicate vmPFC in the regulation of fear- and anxiety-related behaviours in non-clinical populations, it follows that dysfunctional activity within this brain region may be related to symptoms of anxiety disorders in clinical populations. vmPFC dysfunction has been implicated in multiple different types of anxiety disorder despite their heterogeneity, suggestive of common neurobiological substrates mediating aspects of their symptomatology.

1.5.5.1.1 Post-traumatic stress disorder (PTSD)

PTSD is characterised by anxiety and intrusive thoughts following an accident or injury (American Psychiatric Association, 2013). Neurobiological models of PTSD consistently implicate the mPFC – including vmPFC – in the aetiology and pathogenesis of the disease (Brown and Morey, 2012; Shin and Liberzon, 2010). The dominant view is that *decreased* activity in the vmPFC results in impaired inhibition of subcortical limbic structures which become over-active, manifesting as disrupted emotion regulation (Hughes and Shin, 2011). In an influential meta-analysis, Moser and colleagues found evidence for this: PTSD patients show decreased vmPFC activity in a subgenual (sgACC/25 and BA10) region in response to emotional but not neutral scenes (Moser et al., 2015). However, several other meta-analyses suggest a more complex, nuanced picture of the pattern of vmPFC dysfunction in PTSD. A meta-analysis by Thomaes *et al*. evidenced *increased* vmPFC activity in a more rostral (but still subgenual, BA10) region in during the encoding of negative words (Thomaes et al., 2013). Similarly, a recent meta-analysis of resting-state brain function (abrogating confounds of paradigm-related methodological differences) has shown *increased* pgACC/32 activity (extending into dACC and BA10) in patients who develop PTSD compared to trauma exposed controls (Wang et al., 2016). These studies are inconsistent with the dominant view that PTSD patients have impaired emotion regulation owing to a hypoactive vmPFC. The role of the vmPFC in PTSD therefore merits further study.

1.5.5.1.2 Panic Disorder

Panic disorder is characterised by recurrent unexpected panic attacks along with a persistent concern about having future attacks (American Psychiatric Association, 2013). Consistent with studies measuring activity changes following fear/panic induction using pharmacological agents (see **1.2.4.2**), enhanced activity in d/pgACC has been observed in panic disorder patients during imagery of high *vs.* low anxiety situations (Bystritsky et al., 2001). A similar bilateral perigenual region (pgACC/24,32) shows hyperactivity during happy face perception in medicated panic patients (Pillay et al., 2007). Structural studies also suggest decreased grey matter volume in pgACC/32 associated with the disorder (Uchida et al., 2008). Taken together, these studies suggest that activity in pgACC/24,32 is most consistently related to symptoms of panic disorder.

1.5.5.1.3 Social and specific phobia

Increased vmPFC (rostral BA10, pgACC/32) activation in response to negative facial expressions has been reported in patients with social phobia (Amir et al., 2005; Blair et al., 2008). Indeed, a meta-analysis of neuroimaging studies in social phobia suggests the most consistent prefrontal change associated with the disorder is over-activity in a rostral region of vmPFC corresponding to BA10 (Brühl et al., 2014). ^{18}F-FDG PET imaging has also

evidenced decreased metabolism of more caudal regions – including sgACC/25 and caudal BA10 – during resting-state functional imaging in social phobics compared to healthy controls (Evans et al., 2009). Following treatment with tiagabine, activity in an overlapping region increases, suggestive of a role for hypoactive caudal vmPFC in the pathophysiology and treatment response in social phobia.

Studies implicating the vmPFC in *specific* phobias are relatively scarce – meta-analytic approaches instead identify a mid-dACC region associated with symptomatology and treatment response across a variety of fear-evoking stimuli (Ipser et al.). However, Hermann and colleagues found lower vmPFC (BA10) activity in blood phobics *vs.* healthy controls during symptom provocation (Hermann et al., 2007). The relevance of vmPFC dysfunction to blood phobia likely relates to both a failure of emotion regulation but also the critical importance of autonomic dysregulation associated with blood phobias in particular.

1.5.5.1.4 Generalised Anxiety Disorder (GAD)

The literature on neurobiological changes associated with GAD is small. Increased activity across the rostro-caudal extent of the dACC, extending into pgACC/32 and BA10, has been observed when paediatric GAD patients are instructed to attend to their own subjective fear when observing fearful faces (McClure et al., 2007). Similarly, in a mixed cohort of GAD and social anxiety patients, increased intolerance of uncertainty in a decision-making task was associated with increased activity in a large vmPFC region spanning caudal and rostral BA10 (Krain et al., 2008).

Activity within vmPFC subregions has been implicated as a biomarker of successful treatment response as well as a predictor of treatment response in GAD patients. Successful treatment with CBT has been linked with reduced caudal BA10 and sgACC/25 activity when patients are presented with fearful and angry faces (Fonzo et al., 2014). Activity of a more superior region (dorsal aspects of pgACC/32 and dACC/24) seems to successfully predict treatment response in GAD patients to venlafaxine (Whalen et al., 2008). Note that this region is similar to the region of rostral dACC/24 and pgACC/32 whose activity predicts treatment response in depression.

1.6 SUMMARY

Subregions of the primate vmPFC sit at the interface of physiology, affect and cognition, and are consistently implicated in the aetiology and pathogenesis of mood and anxiety disorders. Despite this, we know almost nothing about the causal contributions of these regions to specific symptoms associated with these conditions. In the experiments described in the subsequent chapters, subregions of marmoset vmPFC are pharmacologically manipulated and the effects on baseline physiology (**Chapter 3**), autonomic and behavioural aspects of appetitive processing (**Chapter 4**) and autonomic and behavioural aspects of aversive processing (**Chapter 5**) are measured. The results of these experiments are relevant to preclinical neuroscience and psychiatry alike, as they are the first to show that causal manipulations of NHP vmPFC can induce changes in behavioural and autonomic domains associated with peripheral cardiovascular dysfunction, impaired reward processing and enhanced anxiety.

2 MATERIALS AND METHODS

Abbreviation	Meaning
^{18}F-FDG PET	^{18}Fluorine-fluorodeoxyglucose positron emission tomography
AAV	Adeno-associated virus (DREADDs)
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AP	Anteroposterior
BDNF	Brain-derived neurotrophic factor
BP	Blood pressure
CaMKIIa	Calcium/calmodulin dependent protein kinase promoter (DREADDs)
DHK	Dihydrokainic acid
DREADD	Designer receptor exclusively activated by designer drug
EAAT2	Excitatory amino acid transporter-2
eEF2	Eurkaryotic elongation factor 2
ET	Endotracheal
GABA	γ-aminobutyric acid
GCR	Glucocorticoid receptor
HA	Haemagglutinin (DREADDs)
HI	Human intruder
$hM_{3/4}D_{q/i}$	Protein-engineered muscarinic receptor (DREADDs)
HR	Heart rate
hSyn	Human synapsin promoter (DREADDs)
ICSS	Intra-cranial self-stimulation
IRES	Internal ribosomal entry site (DREADDs)
LM	Lateromedial
LSD	Least squares difference
MAP	Mean arterial pressure
mCitrine	Fluorescent tag (DREADDs)
SEM	Standard error of the mean
vlPFC	Ventrolateral prefrontal cortex

The materials and methods described herein are common to much of the experimental work carried out in this thesis. More specific methods can be found in individual chapters.

2.1 Subjects and Housing

2.1.1 Subjects

Nineteen marmosets (*Callithrix jacchus*, 11 females and 8 males), bred on-site at the University of Cambridge Marmoset Breeding Colony, took part in the studies described in this thesis. They were broadly divided into three cohorts: cohort one for the neutral condition and negative-affect related studies (described in **Chapter 3** and **Chapter 5**), cohort two for the positive-affect related studies (described in **Chapter 4**) and cohort three for studying the effects of peripheral administration of cortisol (**Chapter 6**). Details of the individual subjects taking part in each cohort (including details of specific experiments they took part in) are outlined in **Table 2-1, Table 2-2** and **Table 2-3**. Note that two subjects (Subject 9 and Subject 16) contributed to more than one cohort (Subject 9: cohorts two and three; Subject 16: cohorts one and two).

Subject and sex		Cannulation target	Experimental history	EMOTIONALLY NEUTRAL CONDITION (Chapter 3)	SNAKE EXTINCTION TEST (Chapter 5)	FEAR DISCRIMINATION TEST (Chapter 5)	INTOLERANCE OF UNCERTAINTY (Chapter 5)	
							HI test	HI test with Ketamine
1	M	sgACC/25, pgACC/32	-	✔	✔	✔	✔	✔
2	M	sgACC/25, pgACC/32	-	✔	✔	✔	✔	✔
3	F	sgACC/25, pgACC/32	-	✔	✔	✔	✔	✔
4	M	sgACC/25, pgACC/32	-	✔		✔	✔	✔
5	F	sgACC/25, pgACC/32	-	✔			✔	
6	F	sgACC/25	-				✔	
7	M	sgACC/25	-				✔	

Table 2-1 Subjects and housing: cohort one, for neutral condition and negative-affect related studies. A tick indicates that the subject took part in that phase of the study. Orange shading indicates the subject is deceased, whereas green shading indicates that the subject is still alive. Note that Subject 6 and Subject 7 took part in further experiments following HI testing which are not described in this thesis.

Subject and sex		Cannulation target	Experimental history	FRACTIONATING ANHEDONIA			RESPONSITIVITY TO TREATMENT		CIRCUITRY	HI test
				Appetitive Pavlovian discrimination	Progressive Ratio	Sucrose Preference	Ketamine	Citalopram	PET scanning	
8	F	sgACC/25, pgACC/32	-	✔			✔	✔	✔	
9+	F	sgACC/25, pgACC/32	-	✔						
10	F	sgACC/25, pgACC/32	-	✔			✔	✔	✔	
11*	M	sgACC/25	Appetitive Pavlovian Discrimination						✔	
12	F	sgACC/25	-	✔			✔	✔		
13	F	sgACC/25, pgACC/32	Early iteration of Fear Discrimination test	✔	✔	✔	✔	✔	✔	
14	F	sgACC/25, pgACC/32	Early iteration of Fear Discrimination test	✔		✔		✔		
15*	F	sgACC/25, pgACC/32	Early iteration of Fear Discrimination test		✔	✔				
16*	M	sgACC/25, pgACC/32	Early iteration of Fear Discrimination test		✔	✔				✔

Table 2-2 Subjects and Housing: cohort two, for positive-affect related studies. A tick indicates that the subject took part in that phase of the study. Orange shading indicates the subject is deceased. +Subject 9 lost her cannula implant and went on to contribute to the peripheral cortisol study (**TABLE 2-3**). Post-mortem assessment of the brain did not reveal any significant damage. *Subjects 11, 15 and 16 had telemetry probe failures. Subject 11 had already learnt the appetitive Pavlovian discrimination and so was moved onto PET scanning. Subjects 15 and 16 had telemetry probe failures during fear discrimination training (see following) and so their training was terminated. Subjects 13-16 had varying amounts of fear discrimination training on an earlier iteration of the paradigm, prior to contributing to this cohort. This involved 30-minute sessions five days a week, in which marmosets were presented with auditory cues which were paired either with an aversive loud noise (0.3-0.7s, 115-118dB) or a neutral event (0.5s houselight off). Subjects 13 and 14 failed to learn the discrimination as they did not show a response to the aversive loud noise. Subjects 15 and 16 had telemetry probe failures so were used in behavioural paradigms without telemetric readouts. Subject 16 also contributed to the HI dataset of cohort one (**TABLE 2-1**). Note that any carry-over effects from previous experimental training were controlled for by re-habituating animals to new testing apparatus in between testing cycles.

Subject and sex		Cannulation target	Experimental history	FRACTIONATING ANHEDONIA		ANXIETY
				Appetitive Pavlovian discrimination	Sucrose Preference	HI test
9+	F	sgACC/25, pgACC/32	DREADDs (AAV8-CaMKIIa-HA-hM$_3$D$_q$-IRES-mCitrine)	✔	✔	✔
17	M	sgACC/25, pgACC/32	DREADDs (AAV8-CaMKIIa-HA-hM$_3$D$_q$-IRES-mCitrine)	✔	✔	✔
18	F	sgACC/25, pgACC/32	DREADDs (AAV8-CaMKIIa-HA-hM$_3$D$_q$-IRES-mCitrine)	✔	✔	✔
19	M	sgACC/25, pgACC/32	DREADDs (AAV8-hSyn-HA-hM$_4$D$_i$-IRES-mCitrine)	✔	✔	✔

Table 2-3 Subjects and Housing: cohort three, for peripheral cortisol studies. A tick indicates that the subject took part in that phase of the study. Orange shading indicates the subject is deceased, whereas green shading indicates that the subject is still alive. +Subject 9 lost her cannula implant and moved into this cohort after having contributed to positive-affect related studies shown in **TABLE 2-2**. All of these subjects had also received surgery to infuse a DREADDs viral construct into sgACC/25 – three were infused with the construct AAV8-CaMKIIa-HA-hM$_3$D$_q$-IRES-mCitrine, and one was infused the construct AAV8-hSyn-HA-hM$_4$D$_i$-IRES-mCitrine. DREADDs-related manipulations were carried out at a separate time to cortisol related manipulations. The results of DREADDs manipulations are not reported in this thesis.

2.1.2 Housing

Marmosets were housed in male/female pairs, and males were vasectomised to prevent pregnancies during experimental testing. They were kept in a 12-hour light-dark cycle (lights on at 7 a.m., lights off at 7 p.m.) in a controlled environment of 22 ± 1°C temperature and 50 ± 1% humidity. Their home cages (dimensions: 280 x 120 x 98cm) were stainless-steel backed with high pressure laminate side-walls, plastic trays at the bottom and clear plastic verandas at the top (**FIGURE 2-1**). Each cage contained a nest box, a food tray and wealth of environmental enrichment including swings, ropes and ladders. Animals all had *ad libitum* access to water. Animals in cohort one were fed a varied diet including fruit, rusk, malt-loaf (Soreen, Manchester, UK), peanuts, boiled eggs, sandwiches and weekend treats. Animals in cohort two and three were fed a restricted diet from Sundays to Thursdays consisting of pellets (20g, Special Diet Services, Witham, Essex, UK) with orange on Monday and pellets and carrots on Tuesday to Thursday and Sunday. On Fridays, animals were given rusk (Farley's Rusk, Heinz Foods Ltd., UK), pear and a sandwich with a filling consisting of Nutrica Complan Original (Complan, Trowbridge, Wiltshire, UK), boiled egg, Mazuri Powder (Special Diet Services), multivitamin drops (Abidec; Omega Pharmaceuticals Ltd., London, UK) and vitamin D3 drops (Special Diet Services). On Saturdays, animals were given banana and a sandwich. All procedures were carried out in accordance with the UK Animals

(Scientific Procedures) Act 1986 and the University of Cambridge Animal Welfare and Ethical Review Body. The PPL number for the experiments described in this thesis is P09631465.

Figure 2-1 Subjects and Housing: home cage. The home cage housed the animals and was used for sucrose preference testing and HI testing. **A** Entire home cage, dimensions: 280 x 120 x 98cm. **B** Clear plastic verandas forming the roof of the home cage. **C** Nest box. **D** Food tray. **E** Plastic tray forming the floor of the home cage.

2.2 Surgical Procedures

Animals in cohorts one and two all underwent at least two aseptic surgical procedures: one to implant a telemetric blood pressure monitor into the descending aorta, and one to implant intracerebral cannulae targeting either sgACC/25 alone or both sgACC/25 and pgACC/32. Animals undergoing ^{18}F-FDG PET imaging underwent a third procedure to implant a vascular access port for the administration of radioactive ligands. Telemetric blood pressure monitors were always implanted first, before any behavioural testing.

Animals in cohort three all underwent telemetry surgery. They additionally underwent stereotaxic surgery to infuse a DREADDs viral construct into sgACC/25 (see **Table 2-3**). The results of the DREADDs experiments are not reported in this thesis.

2.2.1 Pre-surgical Procedures

Marmosets were weighed within seven days of surgery. Surgery did not take place if there was a ≥10% decrease in weight compared to the previous week. 24 hours before telemetry surgery, animals were given prophylactic antibiotic treatment with amoxicillin and clavulanic acid (Synulox; 50mg/ml solution; Pfizer, Kent, UK) To minimise the risk of emesis during induction or recovery from anaesthetic, animals were not fed for at least 12 hours prior to surgery.

On the day of surgery, animals were pre-medicated with ketamine hydrochloride (KetaVet; 0.10ml of a 100mg/ml ketamine hydrochloride solution, intramuscular; Henry Schein, Melville, NY, USA) before being given a long lasting non-steroidal anti-inflammatory analgesic (Carprieve; 0.03ml of a 50mg/ml carprofen solution, subcutaneous; Pfizer, Kent, UK). Animals were placed in an incubator at 38°C until fully sedated. Once sedated, marmosets were transferred to a heat mat to maintain body temperature and the hands/feet were shaved so a pulse oximeter could be fitted with adequate contact. Incision sites were also shaved. For telemetry surgery, the abdomen was shaved from the superior border of the femoral triangle to the xiphoid process of the sternum. For cannulation surgery, the scalp was shaved taking care to avoid the ear tufts as these are important social signals. For soloport surgery, the left anterior and posterior triangles of the neck were shaved for visualisation of the internal jugular vein in the carotid canal, together with the back from the iliac crest to a horizontal line at the inferior border of the scapulae when the arms were fully abducted. The animal was then weighed, and this weight was recorded on record sheets.

2.2.2 Anaesthetic Procedures

Once sedated, shaved and weighed the animal was transferred to the surgical suite. An anaesthetic machine (Compact Anaesthesia Systems; VetTech Solutions Ltd., Cheshire, UK) was set up together with a scavenger system. Anaesthesia was induced using a facemask covering the mouth and nose, delivering the inhalational anaesthetic isoflurane (4% in 0.5-1.0 L/min O_2; Novartis Animal Health, Herts, UK). Once the animal was fully anaesthetised (as assessed by an absence of deep-tendon reflexes in ankle extensors and quadriceps muscles), it was intubated. During intubation, a researcher held the marmoset at the angle of the jaw in one hand, whilst the index finger of the other hand hooked onto the marmoset's canine. A second researcher prepared the endotracheal (ET) tube and a Q-tip soaked with the anaesthetic lidocaine (IntuBeaze; Dechra, Shrewsbury, UK). The lidocaine was applied to the epiglottis to relax it, and the ET tube was inserted. The gas supply was then switched from the facemask to the ET tube. A combined pulse-oximeter and capnograph (MicroCap Handheld Capnograph; Oridion Capnography Inc., MA, USA) monitored O_2 saturation (95-100%), heart rate (200-250bpm), breathing rate (12-20 breaths per minute) and end-tidal

CO_2 (35-45mmHg). Core body temperature was monitored using a rectal thermometer (36-38^{o}C, TES-1319 K-type digital thermometer; TES Electrical Electronic Corp., Taipei, Taiwan).

2.2.3 Telemetry Surgery

Marmosets undergoing telemetry surgery were implanted with a transmitter probe which continuously detected and transmitted an arterial BP trace via radio-frequency signals (**FIGURE 2-2A**).

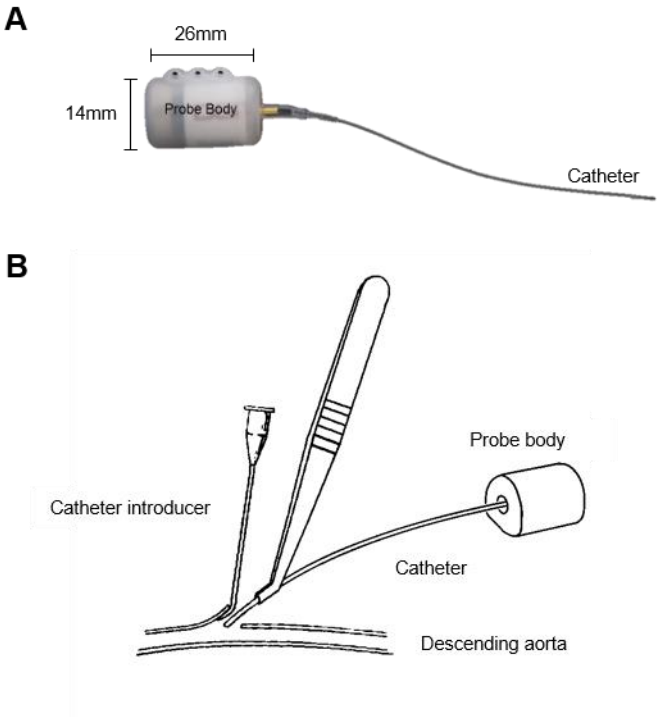


Figure 2-2 Surgical Procedures: telemetry surgery. **A** Telemetry probe. The probe body contains a battery, signal emitter and sensor. The catheter is filled with a non-compressible fluid and bio-compatible gel at the catheter tip. The tip is coated with an anti-thrombogenic film. **B** Implantation procedure. Blood flow in the descending (abdominal) aorta was briefly occluded and a transverse incision was made in the arterial wall. A catheter introducer with a grooved tip was used to lift a flap at the incision site. The catheter was introduced using forceps and guided into the vessel through the groove of the catheter introducer. Once the catheter was in place and an adequate signal was detected, the catheter was secured in place with Vetbond glue and a patch.

Once the animal was under stable anaesthesia, it was placed in a supine position on a sterile drape laid over the heat mat. The upper and lower limbs were secured in place using masking tape to facilitate access to the abdomen. The abdomen was cleaned using chlorhexidine (Chloraprep SEPP applicators; BD, Berkshire, UK) and covered with an Ioban-2 antimicrobial incision drape (3M, St Paul, MN). A laparotomy was performed with a midline incision extending from the xiphoid process to the level of a horizontal line connecting the

anterior superior iliac spines. The skin and rectus sheath was cut using a scalpel blade, and the anterior abdominal muscle wall was cut using scissors. To visualise the abdominal aorta, the intestines were positioned to the sides of the peritoneal cavity and lightly held in place using metal retractors. If the intestines had to be exteriorised, they were kept moist using swabs soaked with sterile saline. The abdominal aorta was exposed from the level of the coeliac trunk to its bifurcation into the common iliac arteries. This portion was separated from the surrounding viscera, fat and connective tissue using two soft pressure swabs. Once the vessel was fully isolated, an 8cm piece of thread was passed under the aorta 1cm above the bifurcation. A haemostat clamped the two ends of the thread, lifting the vessel in preparation for implantation and exerting a small amount of pressure to limit reflux.

To restrict blood flow, an experimenter used an index finger to apply pressure to the aorta at the level of the coeliac trunk, and slight tension was placed on the thread at the base. Pressure was maintained for no more than three minutes to minimise the risk of ischaemia. The vessel was punctured 1cm above the bifurcation using a 23-gauge needle (bent at approximately 60^{o} with the bevelled edge facing upwards). The catheter cuff of the telemetry probe was inserted using a grooved catheter introducer and passed into the aorta until the cuff and a small length of tubing (approximately 1.25cm in total) was contained within the lumen of the vessel (**FIGURE 2-2B**). Once correctly positioned, the experimenter's index finger was gradually released over ten seconds. Approximately 10µL of Vetbond tissue adhesive (3M Animal Care Products, St. Paul, MN, USA) was applied to the puncture site. The glue dried for 30 seconds and the site was monitored for any leakages for a further minute. After the integrity of the seal was established, correct placement of the catheter was then checked using an AM radio (tuned to 600Hz) and a magnet. The magnet was passed over the probe body (resting on the abdominal surface) to activate the probe, and the radio was held directly above the probe body to detect the telemetry signal. A clear fluctuating tone (corresponding to the cardiac cycle) indicated correct placement. After the signal was checked, a small $0.25cm^2$ cellulose patch was placed over the insertion site and a further 10µL of glue was applied to secure the patch and catheter in place.

The thread and retractors were then removed, and the abdominal viscera were moistened with sterile saline. The intestines were gently moved back into position and the probe body was gently rested on top, with the long axis of the probe placed along the midline. The catheter was directed rostrally and kinks were removed. Tabs on the probe body were sutured into the abdominal wall using non-absorbable sutures (Ethilon 3-0 W; Ethicon Inc., Puerto-Rico, USA). After closure of the muscle wall, the signal was checked again and then the probe was switched off using the magnet. It was not turned on again until behavioural testing started after recovery. Finally, the skin was closed with a continuous stitch of

absorbable suture material (Vicryl 3-0 W9444; Ethicon Inc., Puerto-Rico, USA) with the dermis of either side of the suture opposed together and glued using Vetbond. The gas anaesthetic was stopped, and 1.0-2.5ml of sterile saline (volume depended on the length of the surgery) warmed to body temperature was administered to replace fluid loss. Once the animal started to rouse, the ET tube was removed, and the animal was supplied with additional oxygen until it could maintain O_2 saturations of 95-100% independently. The animal was then transferred to an incubator and monitored during recovery.

Once fully recovered, the animal was given 0.25ml Synulox and returned to their home cage with food and water. Post-operative analgesia consisted of 0.1ml Metacam (1.5mg/ml meloxicam; Boehringer Ingelheim, Ingelheim/Rhein, Germany) given orally each morning for three days following surgery. Synulox was administered orally for 5 days following surgery to reduce the risk of infection. The animal was allowed a minimum of 10 days recovery prior to starting behavioural testing.

2.2.4 Cannulation Surgery

Marmosets undergoing cannulation were implanted with 26-gauge double guide cannulae (Plastics One; Roanoke, Virginia, USA) targeting bilateral sgACC/25 alone (7.0mm long, 1.0-1.4mm apart) or both bilateral sgACC/25 and bilateral pgACC/32 (2.0-3.5mm long, 1.0-1.2mm apart).

Once animals were under stable anaesthesia, they were secured in a stereotaxic frame modified for the marmoset (**FIGURE 2-3**, David Kopf Instruments; Los Angeles, California, USA). Ear bars were carefully positioned into the ear canal of each ear to prevent lateral movements and adjusted in the lateromedial (LM) direction until the marmoset was centred in the frame. A mouth bar was placed against the hard palate of the marmoset, and eye bars were positioned in the supraorbital notch of the eye sockets. Lacrilube ointment (Allergan, Bucks, UK) was administered around the eyes and eye bars to prevent the eyes from drying out. The scalp was cleaned using chlorhexidine and an Ioban-2 antimicrobial drape was placed on the scalp. A vertical incision was made along the scalp to expose the skull and skin flaps were held in place using a retractor.

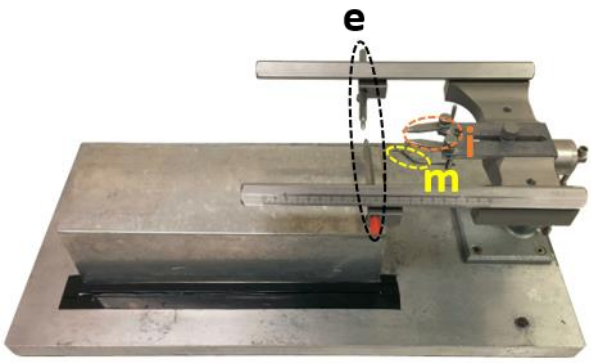


Figure 2-3 Surgical Procedures: Stereotaxic frame for cannulation surgery. The stereotaxic frame was specially modified for the marmoset. The marmoset was secured on the frame using ear bars (**e**), eye bars (**i**) and a mouth bar (**m**).

The coordinate system (in mm) in the stereotaxic set-up used the inter-aural line (at the apex of the ear bars) as the anteroposterior (AP) zero coordinate (positive in the anterior direction) and the superior sagittal sinus at AP +17.5 as the LM zero coordinate (positive to the animal's right). Positioning of guide cannulae to target sgACC/25 and pgACC/32 was adjusted in-situ to account for variation in frontal-lobe size between marmosets. This was achieved by measuring cortical depth at a standard coordinate (AP +17.5, LM -1.5) – a 'depth-check.' The depth-check was done using a smooth dental broach (Micro-Mega, Besancon, France) lowered vertically through the brain. Measurements were taken at the cortical surface with the dura removed, and at the base of the skull. The cortical depth was calculated by measuring the difference between the cortical surface and base. If the depth at this position fell outside the range of 5.8-6.8mm, the AP measurement was adjusted in steps of 0.5mm (anteriorly if the depth was >6.8mm, and posteriorly if the depth was <5.8mm). The depth was re-assessed until it fell within this range. AP alterations were noted, and subsequent AP coordinates were adjusted. The coordinate for pgACC/32 cannulae used the correction from this general depth check. Owing to previous variability in cannula position targeting sgACC/25, an additional depth check was performed at AP +14.0 LM -1.0 (+/- any correction from the general depth check) to more specifically determine the depth around sgACC/25. A depth between 8.9-9.3mm was considered acceptable, and in a similar fashion the AP measurement was adjusted in steps of 0.5mm until the depth fell within this range. The final position of sgACC/25 cannulae was the sum (+/-) of the general depth-check correction and sgACC/25 depth-check correction.

When the location of the cannula was determined, a cortical surface reading was taken with the guide prongs, and the guide cannula could then be lowered into the cortex using the stereotaxic arm. Cannuale targeting sgACC/25 were lowered vertically downwards; cannulae

targeting pgACC/32 were rotated at an angle of 25º towards the anterior in the AP direction. The target depth for sgACC/25 cannulae was 66.7% of the total depth measured, down from the cortical surface. The target depth for pgACC/32 cannulae was 3.5mm from the cortical surface. The guide was lowered until it could be lowered no further (owing to obstruction of adjacent skull). The injector projection lengths (beyond the end of the guide cannulae) for infusions were modified based on the difference between the target depth for the guide and the depth achieved. Final projection lengths consisted of this correction plus a standard length (1mm beyond the end of the guide for sgACC/25; 1.4mm beyond the end of the guide for pgACC/32).

Steel screws (0.80mm, 1/16”; Plastics One) were fastened to the skull surrounding the guide cannulae to facilitate adhesion of cement to the skull. A layer of Super-Bond dental adhesive (Sun Medical, Shiga, Japan) was painted onto the skull surface, providing an optimal bonding surface for the acrylic cement. The acrylic cement was then applied onto the Super-Bond, underneath skull screws and around the guide cannulae. The cement was smoothened using a spatula to prevent any sharp edges. Once the cement had dried, loose skin in-front and behind the cannula implant was sutured using absorbable sutures (Vicryl 3-0 W9444; Ethicon Inc., Puerto Rico, USA). At the end of surgery, marmosets were given 0.18ml of dexamethasone I.M. (3.8mg/ml; Aspen Pharma, Berkshire, UK) to prevent any brain swelling. Stainless steel dummy cannulae were placed into the guide cannulae to maintain patency, and brass or aluminium protective caps were screwed over the top using the thread on the guide cannulae.

After the surgery was completed, the anaesthetic was switched off and the ET tube was removed. The animal was monitored as above, until it maintained O_2 saturations of 95-100% independent of additional oxygen supply. The marmoset was placed in a pre-heated incubator to recover and was monitored until full recovery, after which it was returned to its home cage with food and water. All animals were given the analgesic Metacam for three days following surgery.

2.2.5 Soloport Surgery

Marmosets undergoing ^{18}F-FDG PET imaging were implanted with a subcutaneous soloport system (Solomon Scientific, Skokie, IL, USA) with a catheter in the jugular vein.

Once the marmoset was under stable anaesthesia, a large drape was laid over the heat mat. The marmoset was re-positioned on the heat pad in a prone position with its head facing the surgeon and face turned towards the left side to expose the left triangles of the neck (for targeting of the left jugular vein). The skin was cleaned with chlorhexidine and a sterile marker was used to draw on the position of the vein. A small transverse incision was made

below the tips of the scapulae across the back. Using blunt dissection, a space was created subcutaneously below the incision line which would contain the body of the soloport. The marmoset was then turned onto its side, resting on its hips and arms. The animal's head was tilted to stretch the neck for maximum exposure, and an Ioban-2 antimicrobial drape was cut to cover the neck. A small 2cm incision was made along the line of the sternocleidomastoid muscle taking care not to cut too deeply and damage underlying structures in the carotid canal. The internal jugular vein was visualised, and retractor clips kept the incision site open. Space was made around the vein using blunt scissors to gently separate the connective tissue. Once there was enough space, the flat end of a metal spatula was placed underneath the vessel. The vein was constantly bathed in saline to maintain moisture and to prevent the vein sticking to the spatula. Additional connective tissue enveloping the vein was removed and the spatula was rotated to further free the vein. Further blunt dissection superiorly towards the jaw line ensured there was enough space for the loop of the catheter without any kinks.

Using a hollow skin tunnelling tool, the surgeon tunnelled subcutaneously from the neck incision to the back incision, with a twisting action as the tool advanced. The catheter was threaded through the skin tunnelling tool from the back towards the neck. Once the end of the catheter was visualised in the neck, the soloport and catheter was flushed with 1.0ml heparinised saline (HepSal; Wockhardt UK Ltd., Wrexham, UK) to ensure no blockages had developed during threading of the catheter. With the catheter tip in the neck and the vein isolated on the spatula, a small transverse cut was made from the midline of the vein towards the edge. A blunt 26-gauge needle bent at 45^{o} was inserted into the cut vein. The catheter was inserted to the side of the needle which facilitated smooth entry of the catheter tip into the lumen of the vessel. As the catheter entered the jugular vein, the needle was slowly removed. Once inserted, the catheter was glued in place using 10µL Vetbond and allowed 30 seconds to dry. A 1.0ml syringe with Huber needle attached was then inserted into the soloport and drawn back until the catheter and soloport was filled with blood. A second 1.0ml syringe with Huber needle was used to flush the entire system with a further 1.0ml of HepSal. The catheter tubing was adjusted to ensure a gentle loop back to the soloport and enough slack was left for neck movement. A 0.25cm^2 cellulose patch was then placed over the vessel and 10µL Vetbond was used to glue it in place. The neck and back were then with sutured absorbable sutures (Vicryl 3-0 W9444; Ethicon Inc.).

After the surgery was completed, the anaesthetic was switched off and the ET tube was removed. The animal was monitored as above, until it maintained O_2 saturations of 95-100% independent of additional oxygen supply. The marmoset was placed in a pre-heated incubator to recover and was monitored until full recovery. Once fully recovered, the animal

was given 0.25ml Synulox and returned to their home cage with food and water. Animals were given the analgesic Metacam for three days following surgery, and Synulox was administered orally for 5 days following surgery to reduce the risk of systemic infection. To maintain patency of the soloport system, the soloport was flushed with 0.5ml of HepSal following surgery at regular intervals: +1 day, +3 days, +6 days, +10 days, +15 days and weekly thereafter.

2.3 Behavioural Testing Apparatus

2.3.1 Carry Box

Animals were trained to enter a transparent Perspex carry box (dimensions: 240 x 230 x 200mm) in which they were transported to and from the behavioural testing apparatus. One face of the carry box could be removed and acted as a door. The Perspex carry box could be secured inside a testing chamber using a latch. The marmoset remained inside this box during testing. The door and opposite surface had a circular window (diameter 35mm) together with eight smaller air holes (diameter 15mm).

2.3.2 Testing Chambers

Behavioural testing took place within sound-attenuated automated testing chambers in a dark room. The chambers were lit with LED strips consisting of individually controllable red, blue and green lights which could be turned on together to generate a white 'houselight.' All chambers were equipped with a computer-controlled speaker system through which auditory stimuli could be played. Three video cameras positioned in the test chambers allowed for continuous monitoring of the animal during testing, with the live feed being transmitted to a monitor in another room. Videos were recorded using software (CyberLink PowerDirector, CyberLink Corp., Vaals, ND) for subsequent behavioural scoring. The chambers were adaptable with a modular design – the floor was removeable, such that the same chamber could be used for multiple different behavioural paradigms requiring different sets of equipment (e.g. both appetitive and aversive Pavlovian conditioning). The floor of the apparatus concealed a telemetry receiver (part of the PhysioTel Telemetry System; Data Sciences International, St. Paul, MN, USA) which received a continuous transmission of HR and BP information from telemetry probes implanted into the animals (see **2.6.1**).

In this thesis, two testing chambers (each with a modular design) were used: one for the neutral condition and all Pavlovian conditioning procedures (equipped with removeable foodbox modules [**Chapter 4**] and SmartGlass [**Chapter 5**]), and a second for the progressive ratio (equipped with a touchscreen and milkshake delivery apparatus). Photographs of the first testing chamber are shown in **Figure 2-4A, B** and the second in

FIGURE 2-4C. In both cases, the chamber was controlled by the Whisker control system (Cardinal and Aitken, 2010) and in-house software.

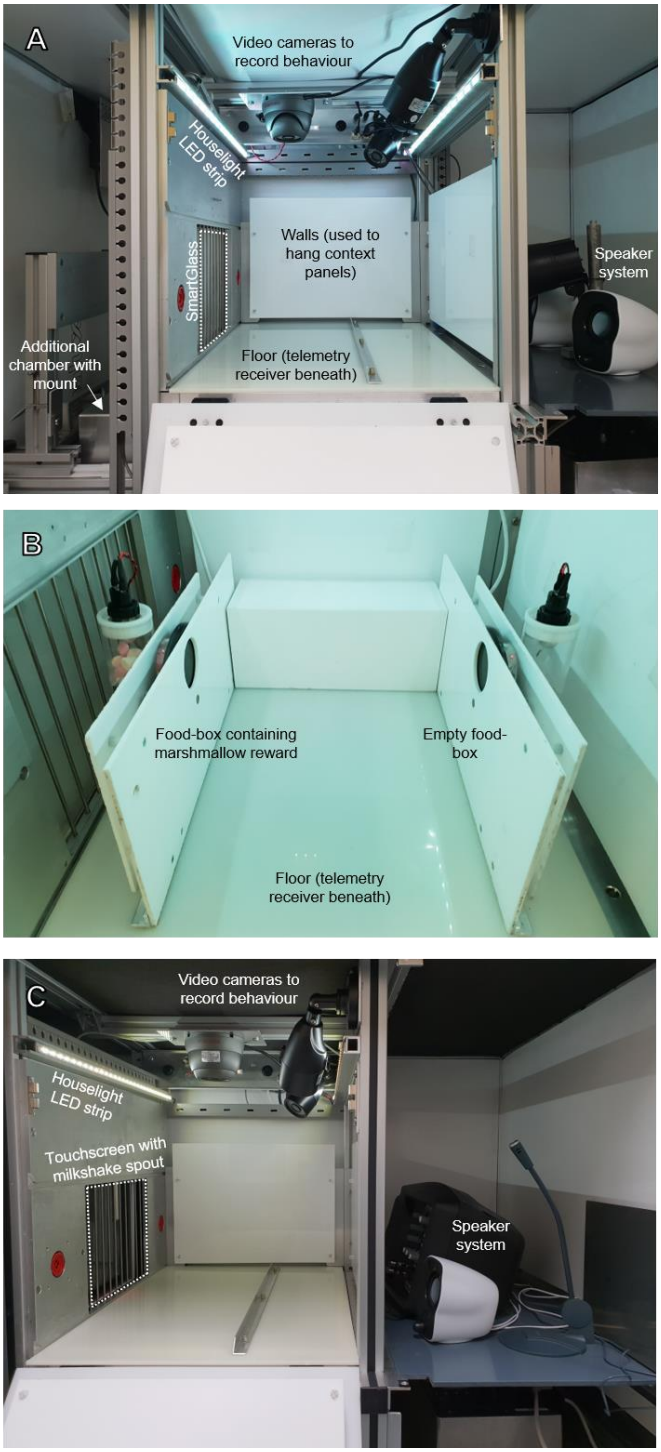


Figure 2-4 Behavioural Testing Apparatus: testing chambers. Two testing chambers were used. **A** First testing chamber for the neutral condition and all Pavlovian conditioning procedures. During the neutral condition (**Chapter 3**), snake extinction conditioning (**Chapter 5**) and fear discrimination testing (**Chapter 5**), the testing chamber appeared as shown. The operation of the SmartGlass is described in **Chapter 5**. During appetitive Pavlovian conditioning testing (**Chapter 4**

and **Chapter 6**), the floor could be removed and replaced with the foodbox apparatus (**B**). **B** Food-box module for appetitive Pavlovian discrimination testing *in-situ* (**Chapter 4** and **Chapter 6**). **C** Second testing chamber for progressive ratio testing (**Chapter 4**).

2.3.3 Home Cage

The sucrose preference test and human intruder (HI) test were carried out in the animals' home cage (**FIGURE 2-1**). During sucrose preference sessions, animals were divided in their home cage, which involved inserting laminated opaque sheets into the cage to divide it into four quadrants. Prior to commencing testing sessions, animals were fully habituated to the dividing procedure by dividing the cage and leaving the animals in one quadrant for 10-15 minutes at a time. Once habituated, sucrose preference testing took place in the top left or top right quadrant. During HI sessions, animals were divided in the top right quadrant.

2.4 DRUG TREATMENTS

The drug used most extensively is this thesis is dihydrokainic acid (DHK), an inhibitor of the excitatory amino-acid transporter-2 (EAAT2) present on astrocytes. This drug and its dose was chosen primarily based on a study in 2012, showing that intra-IL infusions of DHK reduce motivation for ICSS, and importantly do not have gross effects on motor ability nor do infusions induce seizure-like patterns on EEG measurement (John et al., 2012). By inhibiting EAAT2, DHK should increase extracellular glutamate levels and indeed microdialysis studies have shown that administration of DHK increases extracellular glutamate levels (Fallgren and Paulsen, 1996) and intracerebroventricular administration of DHK in the rat increases cFos expression, particularly in IL (Bechtholt-Gompf et al., 2010), consistent with its putative role as an over-activating agent. However, it is important to note that it is not clear which cell populations are having their activity increased by DHK infusions – cFos expression could be in pyramidal output neurons, but also in inhibitory interneurons, and therefore the functional consequences of DHK infusions is more complicated than global over-activation of a brain region. Furthermore, the effect of DHK on glutamate levels will not be restricted to an elevation in glutamate within the synaptic cleft; instead, there will be a global increase in concentration akin to an effect on volume transmission. Thus, the elevated levels of glutamate will affect both synaptic and extra-synaptic metabotropic and inotropic glutamate receptors. These caveats must be borne in mind when interpreting the effects of DHK infusions.

The second method employed to over-activate brain regions in this thesis is a cocktail of two drugs – CGP52432/LY341495 (CGP-LY) – which target $mGlu_{2/3}$ and $GABA_B$ receptors, respectively. These receptors have been shown to negatively modulate glutamate in the prefrontal cortex and hippocampus (Chalifoux and Carter, 2011; Nicoletti et al., 2011) and

combined administration of these two drugs increases *evoked* (but not spontaneous) D-[^{3}H]-aspartate (a non-metabolisable analogue of glutamate) release in ventral hippocampal slice preparations and do not cause nonspecific motor changes when infused into the ventral hippocampus of awake rats (Marrocco et al., 2012). By increasing evoked release, this drug cocktail can be thought of as increasing the sensitivity of brain regions to excitatory inputs.

Muscimol/baclofen is a combination of a $GABA_A$/$GABA_B$ receptor antagonist, and was chosen to silence the activity in particular brain regions as it has been used in the present author's laboratory previously to inactivate vlPFC and OFC (Clarke et al., 2015). These drugs have been used extensively in preclinical research to silence neuronal activity in a myriad of brain regions (although they may not have identical effects – for example, see (Pulman et al., 2012) for different effects of muscimol and baclofen in the accumbens).

The mechanism of action of ketamine is poorly understood and hotly debated. Classically, ketamine is described as an NMDA receptor antagonist but at clinical dose ranges, it has effects on many other receptors (Tyler et al., 2017) – although these effects are weaker than its action at the NMDA receptor (Roth et al., 2013). Hypotheses regarding ketamine's antidepressant action are discussed in more detail in **Chapter 1**, section **1.5.1.5.3**.

Citalopram is an SSRI drug used as first-line (along with sertraline and fluoxetine) in the treatment of moderate to severe depression with consistently demonstrable efficacy (Cipriani et al., 2012; Montgomery and Djärv, 1996). Whilst the antidepressant effects of citalopram take approximately 2-6 weeks to develop, acute doses of citalopram have substantial effects on amygdala responses to fearful faces (Murphy et al., 2009) suggesting that therapeutically relevant actions of SSRIs can occur at much shorter timepoints. Acute intramuscular injections of citalopram have been used in the present author's laboratory to modulate marmoset responses to an uncertain threat in the form of a human intruder (Santangelo et al., 2016).

Naloxone is a non-selective opioid antagonist. Its mechanism of action is not fully understood, although evidence suggests that it antagonises the effects of opioids (both endogenous and exogeneous) through competitive antagonism of μ, κ and δ opiate receptor subtypes, with the greatest affinity for the μ receptor. Clinically, naloxone is given intramuscularly and intranasally to treat opioid overdoses. In both rodents and humans, naloxone administration reduces the consumption of appetitive foodstuffs (Drewnowski et al., 1995; Philopena et al., 1996). However, only sucrose consumption – and not sucrose *preference* (over water) – is affected by naloxone in rats responding for sweet and neutral solutions (Rockwood and Reid, 1982). Likely by devaluation of the outcome, naloxone administration reduces operant motivational responding (as measured by a progressive ratio

schedule of reinforcement) to obtain a sucrose solution in a dose-dependent fashion (Cleary et al., 1996).

Cortisol is the main endogenous glucocorticoid hormone released by the zona fasciculata of the adrenal cortex. The predominant focus when studying the action of cortisol is on the slow, emerging genomic effects of cortisol mediated by the dimersation and nuclear translocation of cytosolic GCR. However, there are also rapid, non-genomic effects of cortisol which occur within 15 minutes of intravenous administration (Strelzyk et al., 2012) – some of this signalling is 'classical'-GCR dependent (blocked by mifepristone) whereas other aspects of this action are insensitive to mifepristone. In both cases, these effects are insensitive to inhibitors of protein synthesis and are therefore non-transcriptional (Dallman, 2005). Acute cortisol administration has effects on human subjective arousal responses and neuroimaging correlates (Sudheimer et al., 2013). The timepoint of one hour used in this thesis probably reflects a combination of rapid, non-genomic and slower, genomic actions of cortisol. Intramuscular cortisol administration at a similar timepoint has been shown to affect maternal behaviours in marmosets previously (Saltzman and Abbott, 2009) and the pre-treatment time/dosage was based on work in this study.

For a summary of doses, pre-treatment times and (where appropriate) infusion parameters of the drugs used in this thesis, see **TABLE 2-4**.

Drug or drug cocktail	Chapter(s)	Mechanism	Route	Dose	Pre-treatment
Dihydrokainic acid (DHK)	3, 4, 5	EAAT2 antagonist	Central infusion	1.35µg/µL Rate of 0.5µL/min	10 minutes
CGP52432/LY341495 (CGP-LY)	4	$GABA_B$ /$mGlu_{2/3}$ receptor antagonist	Central infusion	10ng/µL CGP52432 100pg/µL LY341495 Rate of 0.5µL/min	15 minutes
Muscimol/Baclofen	4	$GABA_A$ /$GABA_B$ receptor agonist	Central infusion	11.4ng/µL muscimol 0.214µg/µL baclofen Rate of 0.25µL/min	25 minutes
Ketamine	4, 5	NMDA receptor antagonist	Intramuscular injection	0.5mg/kg	n/a
Citalopram	4	SSRI	Intramuscular injection	10mg/kg	30 minutes
Naloxone	4	Non-selective opioid receptor antagonist	Intramuscular injection	10mg/kg	10 minutes
Cortisol	6	GCR agonist	Subcutaneous injection	5, 20 and 40mg/kg	60 minutes

Table 2-4 Drug Treatments: Mechanism, route of administration, dose and pre-treatment time for drugs used in experimental manipulations in this thesis. Pre-treatment refers to the time interval between completion of infusion and entry of the animal into the behavioural testing apparatus. All centrally administered drugs were infused over 2mins and injectors were left in place for 1min to facilitate adequate diffusion.

2.4.1 Intracerebral infusions in awake marmosets

Intracerebral infusions in awake marmosets were carried out in a separate minor procedures room. All instruments used were sterile. The area was sprayed down with Anistel (Tristel Solutions, Newmarket, UK) and surfaces were wiped. The syringe pump, syringes and injectors were set-up on a sterile field (**FIGURE 2-5A**). If the drug solution was frozen, it was taken out of the freezer at this point to thaw whilst setting up. Injectors were connected to tubing (0.3mm diameter; VWR International Ltd., UK) fitted to gas-tight 10µL Model 701RN Hamilton syringes (**FIGURE 2-5B**; Hamilton, Reno, NV, USA) attached to an infusion pump (Kd Scientific, Holliston, Massachusetts, USA). The entire infusion system was air-tight and filled with saline. Small air bubbles were drawn up into the set-up and the injector was immersed into drug/vehicle solution and the drug/vehicle was drawn up beyond the position

of the bubble. Small permanent marks were used to check that fluid was moving in the system (**FIGURE 2-5C**).

For all sterile drug treatments, the marmoset was held gently in a researcher's hand. The caps and cannula blockers were removed from the guide and the site was cleaned with 70% ethanol. The injector was inserted into the guide cannulae. Bilateral infusions were carried out over two minutes at a rate dependent on the drug being infused. Following infusion, the injector was left in place for a further minute to allow the drug to diffuse before injector removal. Sterile cannula blockers were placed in the guide cannulae lumen, and sterile brass/aluminium caps were screwed back on before the animal was returned to their home-cage.

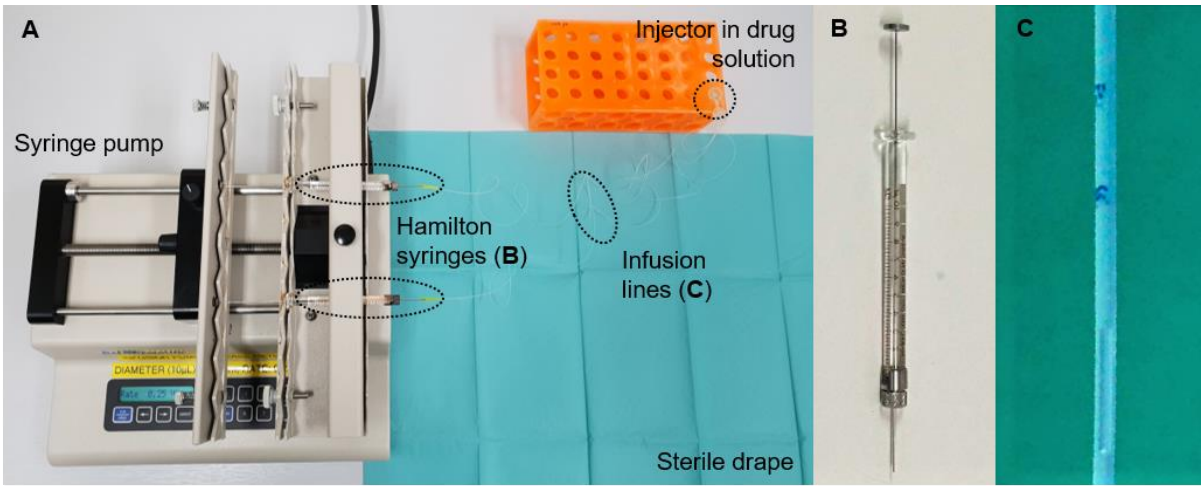


Figure 2-5 Drug Treatments: intracerebral infusions in awake marmosets. **A** Infusion set-up. Hamilton syringes were connected to an injector with PTFE infusion lines. The Hamilton syringes were mounted on a syringe pump with an adjustable rate. **B** Hamilton syringe. **C** Infusion line. Prior to an infusion, the syringes and lines were filled with 0.9% sterile saline. A bubble was manually drawn up, and then drug (or vehicle) was drawn up such that the fluid after the bubble was the substance to be infused. The bubble was marked with permanent marker. This was used to assess fluid movement – as can be seen, the bubble has moved beyond the initial markings.

2.4.2 Systemic drug treatments

Monkeys were taken into a separate room, held by a researcher. For intramuscular injections, the lateral aspect of the thigh was cleaned with alcohol before injection. An insulin syringe was used to inject at 45^{o} to the surface of the skin into the body of the quadriceps muscle. No more than 0.1ml of fluid was injected via this route at any one time.

For subcutaneous injections, an area of skin in between the shoulder blades was cleaned with alcohol before injection. This skin was pinched upwards and an insulin syringe was used

to inject at a 20º angle. The area was massaged briefly to encourage the distribution of fluid beneath the skin. The volume injected via this route was 0.8ml/kg.

2.5 Salivary Cortisol Sampling

Salivary cortisol samples were taken using a cotton bud. When the cotton bud was soaked with saliva, the end was snipped off and placed in an Eppendorf tube and sealed. The Eppendorf tubes were closed and placed at -20ºC for storage, for no more than one month. The samples were analysed using the Salimetrics® salivary cortisol assay (Stratech, Newmarket, UK).

'Pre'- and 'post'-manipulation samples were always taken as a pair and analysed together as part of the same batch to minimise the effect of any systematic variation between batches. The 'pre' sample was typically taken before a testing session (e.g. during an infusion or injection), whereas the 'post' sample was taken after. Previous work in NHPs has shown that salivary cortisol responses in response to an ACTH injection stressor start at 15 minutes and peak at 45 minutes (Heintz et al., 2011) – meaning that salivary cortisol samples adequately reflect fluctuations in circulating cortisol over the typical length of a testing session (between 15-30 minutes). Within single studies, salivary samples were taken at the same time of day (within one hour) to minimise the influence of circadian rhythms.

2.6 Data Acquisition and Preliminary Analysis

2.6.1 Telemetry data collection and analysis

BP data were continuously transmitted by the implanted probe to a receiver in the behavioural testing chamber. Each component was part of the PhysioTel system (Data Sciences International, St. Paul, MN, USA), using short range telemetry to record data from untethered animals (**Figure 2-6**). An ambient pressure reference monitor (APR-1) continuously recorded ambient pressure. The absolute pressure measured by the telemetry probe and the ambient pressure data were collated using a matrix data acquisition system (MX-2) together with interface software to transfer the recorded data to a Spike2 datafile (version 8.12; Cambridge Electronic Design [CED], Cambridge, UK) on an acquisition computer. The acquisition computer computed the animal's BP by comparing the absolute pressure measured by the probe and the ambient pressure measured by the APR-1. This could be used for offline analysis.

Outliers and recording failures were removed (values >200mmHg or <0mmHg, and other abnormal spikes) and gaps were treated as missing values, although data collection was highly reliable overall. Systolic and diastolic events were extracted as local maxima and minima for each cardiac cycle. MAP was calculated for each cycle using the following

formula: MAP = diastolic BP + $\frac{1}{3}$(systolic BP – diastolic BP). HR values were derived from inter-beat intervals.

2.6.2 Behavioural Analysis

Behavioural data were collected via video recording systems and scored offline by an experimenter and research assistant. Refer to individual chapters for more detailed behavioural methodologies.

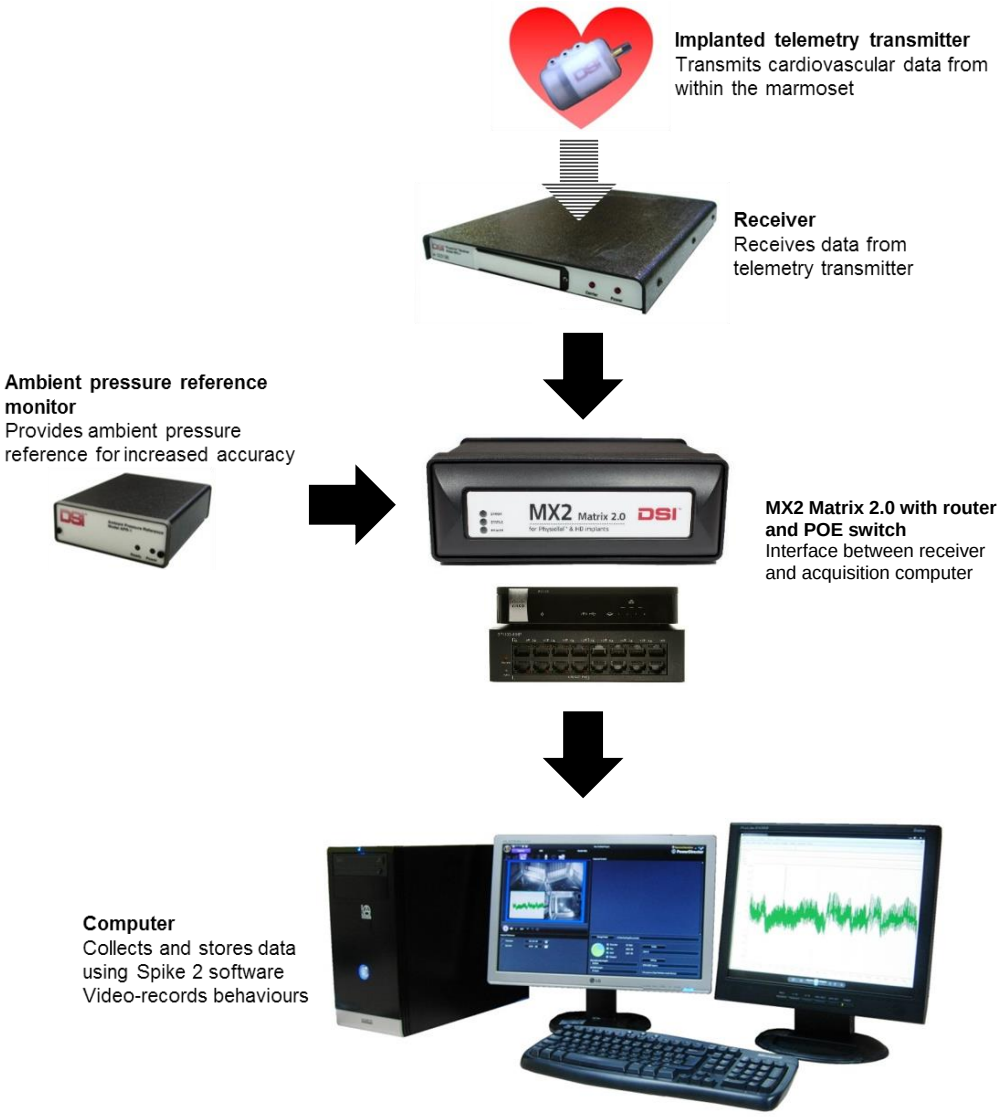


Figure 2-6 Data Acquisition and Preliminary Analysis: telemetry data acquisition and analysis. An MX2 matrix system gathered pressure information from the animal via a receiver, and ambient pressure using an ambient pressure reference monitor. Absolute and ambient pressure information was interfaced to the data acquisition computer which could be used to calculate the animal's BP and generate a continuous telemetric readout.

2.7 Statistical Analysis

Data were inputted into GraphPad Prism v8.00.178 for Windows (GraphPad Software, La Jolla, CA) for statistical analysis. Significance was set at α=0.05 in all cases. The design of all studies was repeated-measures, within-subject. Analyses were typically carried out as follows:

- **Single group of data:** summarised with mean ± SEM. If appropriate, a one sample *t*-test was used to compare data to a hypothetical mean.
- **One factor with two levels**: analysed with a two-tailed paired *t*-test.
- **One factor with three or more levels**: analysed with a one-way repeated-measures analysis of variance (ANOVA).
- **Two factors**: analysed with a two-way repeated-measures ANOVA.
- **Three factors**: analysed with a three-way repeated-measures ANOVA.

Parametric tests were conducted as the sample size was too small to detect violations in normality, and magnitudes of effects were generally similar across animals. In most cases, *post-hoc* tests were corrected for using Sidak's correction for multiple comparisons. When planned comparisons were made based on data from pre-existing literature (for instance, the effects of naloxone on sucrose consumption), *post-hoc* p-values were computed from Fisher's Least Squares Difference (LSD) test and remain uncorrected. For precise details of the statistical analyses conducted, refer to individual chapters.

2.8 *Post-mortem* Assessment of Cannula Placement

Animals were pre-medicated with ketamine hydrochloride before being euthanized with pentobarbital sodium (Dolethal; 1ml of a 200mg/ml solution, i.e.; Merial Animal Health, Essex, U.K.). Animals were then perfused transcardially with 500ml 0.1M phosphate-buffered saline, followed by 500ml of 4% paraformaldehyde fixative solution over approximately 15 minutes. The brain was removed and left in the 4% paraformaldehyde fixative solution overnight before being transferred to 30% w/v sucrose solution for at least 48 hours. Brains were then sectioned on a freezing microtome (coronal sections; 40 or 60µm), mounted on slides and stained with cresyl-violet. The sections were viewed under a Leitz DMRD microscope (Leica Microsystems, Wetzlar, Germany). The cannula locations for each animal were schematized onto drawings of standard marmoset brain coronal sections and composite diagrams were then male to illustrate the extent of overlap between animals.

3 CARDIOVASCULAR CHANGES INDUCED BY OVER-ACTIVATING PRIMATE SGACC/25 BUT NOT PGACC/32

Abbreviation	Meaning
ANOVA	Analysis of variance
BA	Brodmann area
BOLD	Blood oxygen level dependent
BP	Blood pressure
CAN	Central autonomic network
CSI	Cardiac sympathetic index
CVI	Cardiac vagal index
DMN	Default mode network
EAAT2	Excitatory amino acid transporter-2
HPA	Hypothalamo-pituitary-adrenal
HR	Heart rate
HRV	Heart rate variability
IBI	Inter-beat interval
IL	Infralimbic (cortex)
MAP	Mean arterial pressure
NHP	Non-human primate
pgACC	Perigenual anterior cingulate cortex
PL	Prelimbic (cortex)
RMSSD	Root mean squared standard deviation
sgACC	Subgenual anterior cingulate cortex
vmPFC	Ventromedial prefrontal cortex
VNS	Vagal nerve stimulation

3.1 Abstract

Subregions of the ventromedial prefrontal cortex (vmPFC) are appreciated as critical structures in the regulation of both the cardiovascular system and HPA axis, and dysfunctional activity within the vmPFC – together with dysregulated cardiovascular and endocrine physiology – has been implicated in disorders associated with enhanced negative emotion, including depression and anxiety. However, whether these changes are causally linked is unknown. Here we show for the first time that over-activity in sgACC/25 – but not pgACC/32 – profoundly alters cardiovascular function in an emotionally neutral condition. This same manipulation has no effect on HPA axis activity as measured by salivary cortisol concentration. Specifically, sgACC/25 over-activity is associated with reduced HRV and reduced vagal tone, changes which are commonly observed in depression, anxiety and psychopathology more generally. The data presented here are the first to elucidate a causal link between elevated activity in sgACC/25 and cardiovascular changes characteristic of psychiatric disease and suggest that sgACC/25 is a critical component of the neurobiological 'link' between mood disorders, anxiety disorders and cardiovascular disease.

3.2 Introduction

Dysfunctional cardiovascular (Carney et al., 2001; Khawaja et al., 2009) and endocrine (Keller et al., 2017) activity is a physiological hallmark of both mood and anxiety disorders (Carney et al., 2001; Chalmers et al., 2014; Faravelli et al., 2012; Keller et al., 2017; Khawaja et al., 2009). Beyond over-activity in vmPFC subregions being implicated in the diagnostic symptoms of these disorders (Drevets et al., 2008b; Hamilton et al., 2011a; Harrison et al., 2009; Mayberg, 1997; Mayberg et al., 2005), these same regions have an involvement in homeostatic cardiovascular and HPA axis regulation. A large region of rostral and caudal vmPFC – including both sgACC/25 and pgACC/32 – has been implicated in the central regulation of autonomic function (Beissner et al., 2013), termed the central autonomic network (CAN) (Loewy and Spyer, 1990). The CAN is a critical regulatory system involved in visceromotor and neuroendocrine control to maintain a constant internal milieu (Benarroch, 1993). Given these functions – together with the implication of over-activity in these areas in the symptoms of mood/anxiety disorders – it begs the question as to whether over-activity within vmPFC subregions such as sgACC/pgACC contributes to aspects of physiological dysfunction associated with these disorders.

There are indications from several lines of correlational neuroimaging work that human sgACC/25 and pgACC/32 are associated with different aspects of autonomic function. SgACC/25 has been linked to modulation of parasympathetic activity at rest owing to its connectivity to components of the DMN (Hamilton et al., 2011a), together with a strong positive association of activity within this region to the high frequency component of HRV

(Allen et al., 2015). Studies in humans have also implicated activity in sgACC/25 in parasympathetic regulation during emotional processing (Lane et al., 2013). PgACC/32 shows strong inverse correlations with HR during handgrip tasks and situations of emotional stress (Gianaros and Wager, 2015; Goswami et al., 2011; Wager et al., 2009), such that decreased activity within pgACC/32 is associated with increased HR. This suggests that activity within pgACC/32 provides tonic, top-down inhibition of HR which is released when cardiac output needs to increase. However, a definitive role for these subregions in cannot be determined from these studies alone: beyond a (potentially differential) role for pgACC/32 and sgACC/25 in cardiovascular modulation, the causal roles of these regions remains unclear based on the results of correlative neuroimaging studies alone.

Interventional studies in rodents provide further insight into the differential roles of the putative homologues of sgACC/25 and pgACC/32 – IL and PL respectively – in cardiovascular regulation. IL and PL constitute rodent 'visceral motor cortex' (Terreberry and Neafsey, 1983). Anatomical tract-tracing studies show that whilst IL directly projects to basomedial amygdala, hypothalamic nuclei and brainstem autonomic control centres, PL projects to insula, claustrum, thalamus and basolateral amygdala (Vertes, 2004). These connectivity patterns suggest that IL is a major cortical autonomic motor structure, whereas the function of PL is integrative, acting as a site of convergence for limbic, cognitive and autonomic inputs. Functional studies have tended to support this: stimulation of IL induces baseline physiological changes, whereas stimulation of PL has no baseline effect but regulates autonomic changes induced by amygdala/hypothalamic stimulation (Al Maskati and Zbrożyna, 1989). Recent work has identified PL opioid and angiotensin receptors as being critical in generating the cardiovascular components of the stress response induced by acute restraint (Brasil et al., 2018; Fassini et al., 2014), further supporting a role for PL in the regulation of autonomic function during situations of arousal. Having said this, IL is not limited to a role in baseline cardiovascular function: IL has also been related to cardiovascular responses during stress, but in contrast to PL, it is proposed to attenuate these responses (Müller-Ribeiro et al., 2012).

Despite being apparently anatomically homologous (Haber, 2016), whether rodent PL/IL regions are functionally analogous to human pgACC/32 and sgACC/25 remains uncertain. Interventional pharmacological manipulations carried out in marmosets in the present author's laboratory have shown that sgACC/25 inactivations have profound cardiovascular effects in an emotionally neutral condition to increase HRV and increase vagal tone, whereas inactivations of pgACC/32 have an effect limited to a modest elevation in BP (Wallis et al., 2017). However, the consequences of over-activity in these subregions – an arguably more

translationally relevant change – has yet to be investigated. Furthermore, the consequences of over-activity on neuroendocrine function remains unclear.

The aim of this study was to investigate the causal relationship between over-activity in sgACC/25 or pgACC/32 to cardiovascular and endocrine changes in an emotionally neutral condition. In this condition, animals were habituated to and tested in a highly familiar testing apparatus. Over-activation of sgACC/25 was induced using the drug DHK – an EAAT2 inhibitor. This drug is particularly relevant as reduced EAAT2 expression has been measured in post-mortem brain tissue of depressed patients (Miguel-Hidalgo et al., 2010) and in animal models of depression (Zink et al., 2010). Using telemetric monitoring, the effects of over-activation of sgACC/25 and pgACC/32 on HR, MAP and HRV were determined. Salivary cortisol samples were taken before and after the session to measure the effects on HPA axis function as indexed by cortisol output.

3.3 METHODS

3.3.1 Subjects

Five marmosets (two females, three males) took part in this study. These marmosets were Subjects 1-5 of cohort one, described in **2.1.1 SUBJECTS**. The marmosets were housed and cared for as described in **2.1.2 HOUSING**.

3.3.2 Surgical Procedures

Five marmosets underwent two surgical procedures prior to taking part in the study: one to implant a telemetric blood pressure probe and one to implant intracerebral cannulae targeting sgACC/25 and pgACC/32 (see **2.2 SURGICAL PROCEDURES**).

3.3.3 Behavioural testing apparatus and paradigms

Animals were placed inside a Perspex carry box inside a testing chamber as described in **2.3 BEHAVIOURAL TESTING APPARATUS**. Briefly, the testing chamber consisted of three white walls, a white plastic floor and a telemetry receiver beneath the floor.

3.3.3.1 Habituation sessions

After recovery from telemetry and cannulation surgery, marmosets were trained to enter a Perspex carry box in which they were transported to the apparatus. During habituation sessions, monkeys were placed inside the testing chamber with the houselight turned on. Initial habituation sessions were approximately five minutes long, with the length gradually extended to a maximum of 20 minutes over a period of 5-7 days. The total number of habituation sessions depended on the individual animal's rate of acclimatisation to the apparatus. The animal was judged to have habituated when HR reached a consistent, stable level across two days (± 10%) and based on the experimenter's assessment of the animal's behaviour (relaxed and still with a non-vigilant posture). Two animals from the cohort of five failed to habituate to sessions longer than 15 minutes (showing agitated behaviours in the last 5 minutes, including excessive movement and grooming in the box), so their sessions were capped just short of this length (12 minutes). This meant that data were analysed over minutes 1-10 of test sessions. Minute 0 was excluded, as during this minute animals re-acclimatise to the apparatus following transportation by the experimenter. Minutes 1-10 were analysed because (i) habituation data were available for all five animals over minutes 1-10 and (ii) all animals appeared calm from behavioural and cardiovascular readouts over this period. Habituation sessions meant that the testing chamber became as emotionally neutral as possible, such that the cardiovascular/endocrine outputs being measured were closely reflecting an 'at rest' state. However, the context cannot be assumed to be completely neutral. For example, the testing chamber may act as a mild negative context as animals are confined to a smaller area, away from their partner.

3.3.3.2 Mock infusion sessions

After animals were habituated to standard habituation sessions, mock infusion sessions were carried out. In these, animals were handled by an experimenter prior to the testing session as if they were having an intracerebral infusion. This meant animals became acclimatised to the infusion procedure prior to actual experimental manipulations. Animals were deemed to have acclimatised when no discernible cardiovascular or behavioural effects of the mock infusion could be observed in the following 20-minute testing session. This typically took 1-2 sessions.

3.3.3.3 Experimental manipulation sessions

Experimental manipulation sessions were identical to mock infusion sessions, except prior to the testing session animals were infused with either saline vehicle or DHK into sgACC/25 or pgACC/32 (see **3.3.4**).

3.3.4 Drug treatments

Within-subject intracerebral drug treatments were carried out as described in **2.4.1 INTRACEREBRAL INFUSIONS IN AWAKE MARMOSETS**. The pharmacological compounds used in experimental manipulations in this study were: 0.9% saline (vehicle control) and DHK (an EAAT2 inhibitor). Over-activation using this method is of particular relevance, since EAAT2 shows reduced expression levels in post-mortem cerebral tissue samples of depressed patients (Choudary et al., 2005b; Miguel-Hidalgo et al., 2010) and in animal models of depression (Zink et al., 2010). EAAT2 inhibition results in over-activation through inhibition of glutamate reuptake.

3.3.5 Salivary cortisol sampling

Salivary cortisol samples were taken and processed as described in **2.5 SALIVARY CORTISOL SAMPLING**. Specifically, a salivary sample of cortisol was taken during the infusion as a 'pre' sample before the test session. After the animal had finished the test session, a second 'post'-test salivary sample of cortisol was taken.

3.3.6 Data acquisition and preliminary analysis

Telemetry data, including MAP and HR values, were collected as described in **2.6.1 TELEMETRY DATA COLLECTION AND ANALYSIS**. The following HRV measures were also quantified:

- The root mean squared standard deviation (RMSSD) of the time difference between consecutive IBIs (higher values indicate more variability) as this metric has been shown to be resistant to changes in respiratory sinus arrhythmia (Shaffer and Ginsberg, 2017);

- Indices of parasympathetic (cardiac vagal index, CVI) and sympathetic (cardiac sympathetic index, CSI) activity derived from Poincare plots of successive IBIs (as described in (Toichi et al., 1997)); and
- CSI/CVI ratio.

3.3.7 Statistical analysis

3.3.7.1 Cardiovascular data

As mentioned above, data were analysed over minutes 1-10 of test sessions. The 0th minute was excluded to allow time for acclimatisation to the apparatus after transport. Minutes 1-10 were chosen because (i) habituation data were available for all five animals over minutes 1-10 and (ii) all animals appeared calm from behavioural and cardiovascular readouts over this period.

To illustrate successful habituation to the apparatus, a one-way ANOVA was conducted to measure the effect of session on HR and MAP responses in the first, penultimate and final habituation sessions. Planned comparisons were carried out between the first *vs.* penultimate and first *vs.* final habituation sessions using Fisher's LSD test (p values uncorrected).

The main analyses were conducted on data obtained within individual regions, since the hypothesis in question was whether sgACC/25 and/or pgACC/32 contribute to cardiovascular dysfunction *independently* of one another. To do this, sgACC/25 over-activation was compared to infusions of saline (vehicle) control on individual measures using two-tailed paired *t*-tests. PgACC/32 over-activation was compared to infusions of saline vehicle in the same way. Second-by-second HR and MAP values across entire sessions were analysed with an ANOVA performed with R version 3.4.1 using the lme4 package (Bates et al., 2014) for linear mixed-effects modelling, with statistical tests from the lmerTest package (Kuznetsova et al., 2016) using type III sum of squares with the Sattherwaite approximation for degrees of freedom, reported to the nearest integer. Fixed effect factors included treatment (control *vs.* over-activation) and time; the random effect factor was subject (individual marmosets) to take into account inter-individual differences between animals.

An additional set of analyses directly compared the two regions, to determine whether there was a differential effect of sgACC/25 and pgACC/32 over-activation on individual measures of cardiovascular activity. This was done using two-way repeated measures ANOVAs of the form $M_2 \times A_2$ where *M* is a factor with two levels (manipulation: control *vs.* over-activation) and *A* is a factor of two levels (area: sgACC/25 *vs.* pgACC/32).

3.3.7.2 Salivary cortisol samples

A ratio of salivary cortisol measured from 'post' samples compared to 'pre' samples was calculated, and the 'post':'pre' ratio was compared between control and over-activation conditions. In addition to the precautions taken by testing animals at approximately the same time of day, the calculation of this ratio controls for day-to-day fluctuations in absolute levels of salivary cortisol. Additionally, the ratio values for individual manipulations were compared to a hypothetical value of 1.0 using a one-sample *t*-test to determine if the ratios significantly differed from a value of 'no change.'

3.3.8 Post-mortem histological processing

Of the cohort of five animals used in this study, four are still alive. Brain sections were prepared and visualised as described in **2.8 POST-MORTEM ASSESSMENT OF CANNULA PLACEMENT**.

3.4 RESULTS

3.4.1 Post-mortem assessment of cannula placement

Histological analysis revealed that cannulae successfully targeted pgACC/32 and sgACC/25 in the one animal for which post-mortem tissue is currently available (**FIGURE 3-1**). The other animals constituting this cohort are still alive.

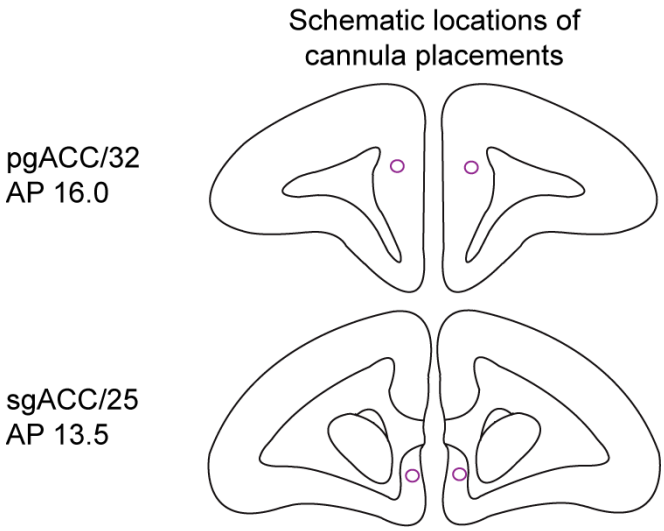


Figure 3-1 Cannula placements. Location of pgACC/32 and sgACC/25 cannulae for the animal where post-mortem placements are available.

3.4.2 Habituation to the testing apparatus

The number of habituation sessions required before experimental manipulations took place was 28 ± 9 (mean ± SEM). There was a significant reduction in HR and MAP across the first, penultimate and final habituation sessions indicating successful habituation as measured by these cardiovascular indices (**FIGURE 3-2A, B**). The mean ± SEM change in HR from first to last session was 116 ± 13bpm, and the mean ± SEM change in MAP was 16 ± 6mmHg.

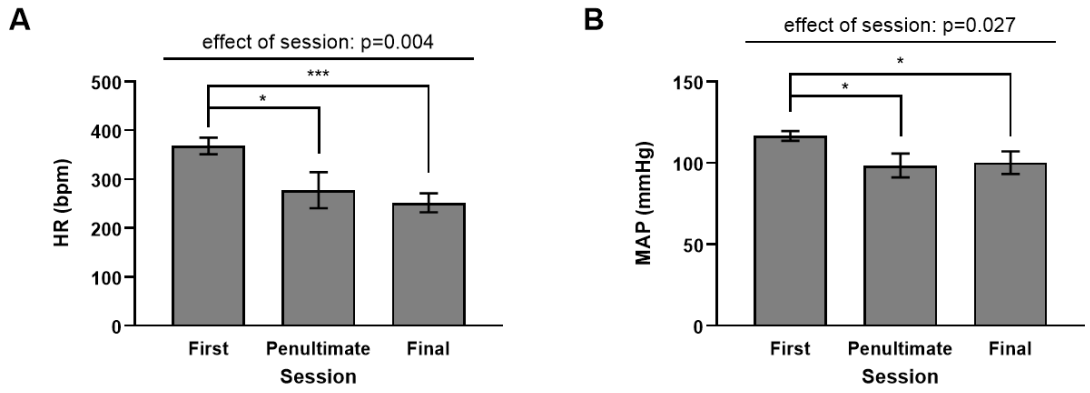


Figure 3-2 Mean HR and MAP responses across the first, penultimate and final habituation sessions prior to experimental manipulations. Relevant graphs show mean ± SEM. N=5. **A** There was a significant difference in HR across these sessions (effect of session: $F_{1.439,5.755}$=18.26, p=0.004), with planned comparisons using Fisher's LSD test revealing a significant difference between first *vs.* penultimate (p=0.021) and first *vs.* final (p<0.001) sessions. **B** There was a significant difference in MAP across these sessions (effect of session: $F_{1.289,5.156}$=8.80, p=0.027), with planned comparisons using Fisher's LSD test revealing a significant difference between first *vs.* penultimate (p=0.028) and first *vs.* final (p=0.047) sessions.

3.4.3 SgACC/25 over-activation profoundly alters baseline cardiovascular activity, but pgACC/32 over-activation has no effect

Over-activation of sgACC/25 (n=5) had no effect on baseline MAP (**FIGURE 3-3A**) but significantly increased HR (**FIGURE 3-3B**). Over-activation significantly reduced HRV as measured by RMSSD (**FIGURE 3-3C**) and altered sympathetic-parasympathetic balance manifesting as an increase in CSI/CVI ratio (**FIGURE 3-3D**). This change was driven predominantly by a significant reduction in the CVI (**FIGURE 3-3E**); CSI showed a tendency to increase, but this was not significant (**FIGURE 3-3F**).

The 10-minute analysis window (60-660s) was split into 600, 1s time-bins and HR/MAP values were calculated within each bin for control and over-activation conditions. Second-by-second MAP values are plotted in **FIGURE 3-3G**, and second-by-second HR values in **FIGURE 3-3H**. SgACC/25 over-activation did not significantly increase MAP, but significantly raised HR throughout the analysis window.

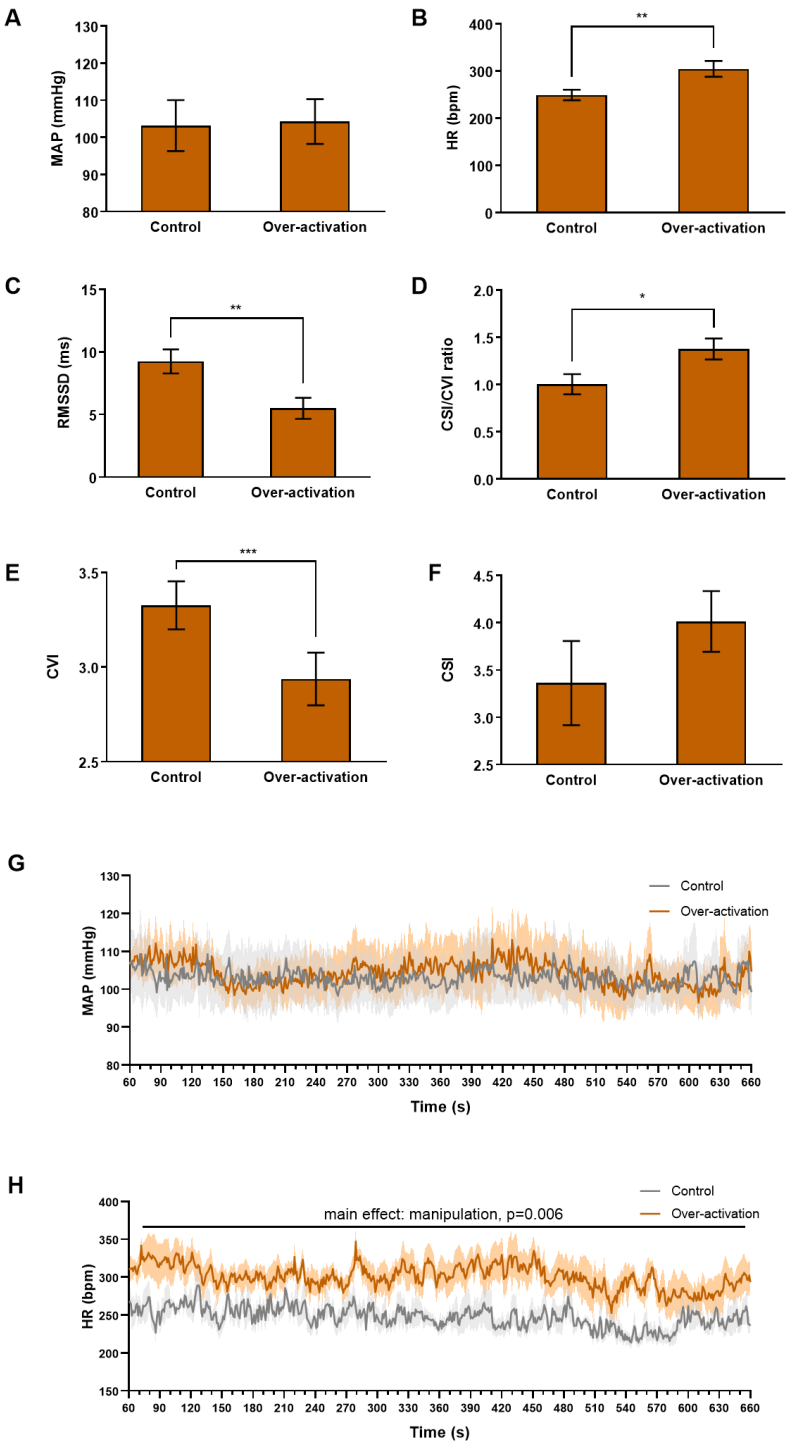


Figure 3-3 SgACC/25 over-activation had profound effects on baseline cardiovascular function. Relevant graphs show mean ± SEM. P values for **A-F** reported from the two-tailed paired *t*-tests. N=5. **A** SgACC/25 over-activation had no effect on baseline MAP (p=0.920). **B** SgACC/25 over-activation significantly increased baseline HR (p=0.008). **C** SgACC/25 over-activation significantly reduced baseline HRV as measured by the RMSSD of successive IBIs (p=0.003). **D** The balance of sympathetic:parasympathetic input to the heart was shifted as indicated by an increase in the CSI:CVI ratio (p=0.032). **E** The change in CSI:CVI ratio appeared to be driven by a reduction in CVI (p<0.001). **F** There was less consistent effect to increase CSI (p=0.100). **G** From

second-by-second MAP values plotted across the entire 10-minute analysis window, whilst there was a time dependent effect of sgACC/25 over-activation, it had no effect to systematically change MAP (manipulation × time, $F_{1,5941}$=24.8, p<0.0001; effect of manipulation, p=0.790). **H** From second-by-second HR values plotted across the entire analysis window, it is evident that sgACC/25 over-activation systematically increases HR (manipulation × time: F<1, NS; main effect of manipulation: $F_{1,4}$=27.55, p=0.006).

Over-activation of pgACC/32 (n=5) using DHK had no effect on any cardiovascular measure (**FIGURE 3-4A-F**). Second-by-second MAP (**FIGURE 3-4G**) and HR (**FIGURE 3-4H**) plots confirm a lack of effect on MAP and HR measures throughout the session.

3.4.4 Differential effects of sgACC/25 and pgACC/32 over-activation on baseline cardiovascular activity

We additionally directly compared the effects sgACC/25 *vs.* pgACC/32 on individual measures, to determine whether over-activation of these regions differentially affected indices of cardiovascular activity as indicated by a significant manipulation x treatment interaction:

- **HR**: There was evidence of a significant differential effect of sgACC/25 *vs.* pgACC/32 over-activation on HR (manipulation × area, $F_{1,4}$=11.34, p=0.028).
- **RMSSD**: There was a trend towards a differential effect of sgACC/25 *vs.* pgACC/32 over-activation on RMSSD (manipulation × area, $F_{1,4}$=5.14, p=0.086).
- **CSI/CVI ratio**: There was a trend towards a differential effect of sgACC/25 *vs.* pgACC/32 over-activation on CSI/CVI ratio (manipulation × area, $F_{1,4}$=6.26, p=0.067).
- **CVI**: There was evidence of a significant differential effect of sgACC/25 *vs.* pgACC/32 over-activation on CVI (manipulation × area, $F_{1,4}$=13.08, p=0.022).

There was no evidence of a differential effect on other cardiovascular measures, namely MAP (manipulation × area, F<1, NS) and CSI (manipulation × area, $F_{1,4}$=2.88, p=0.165).

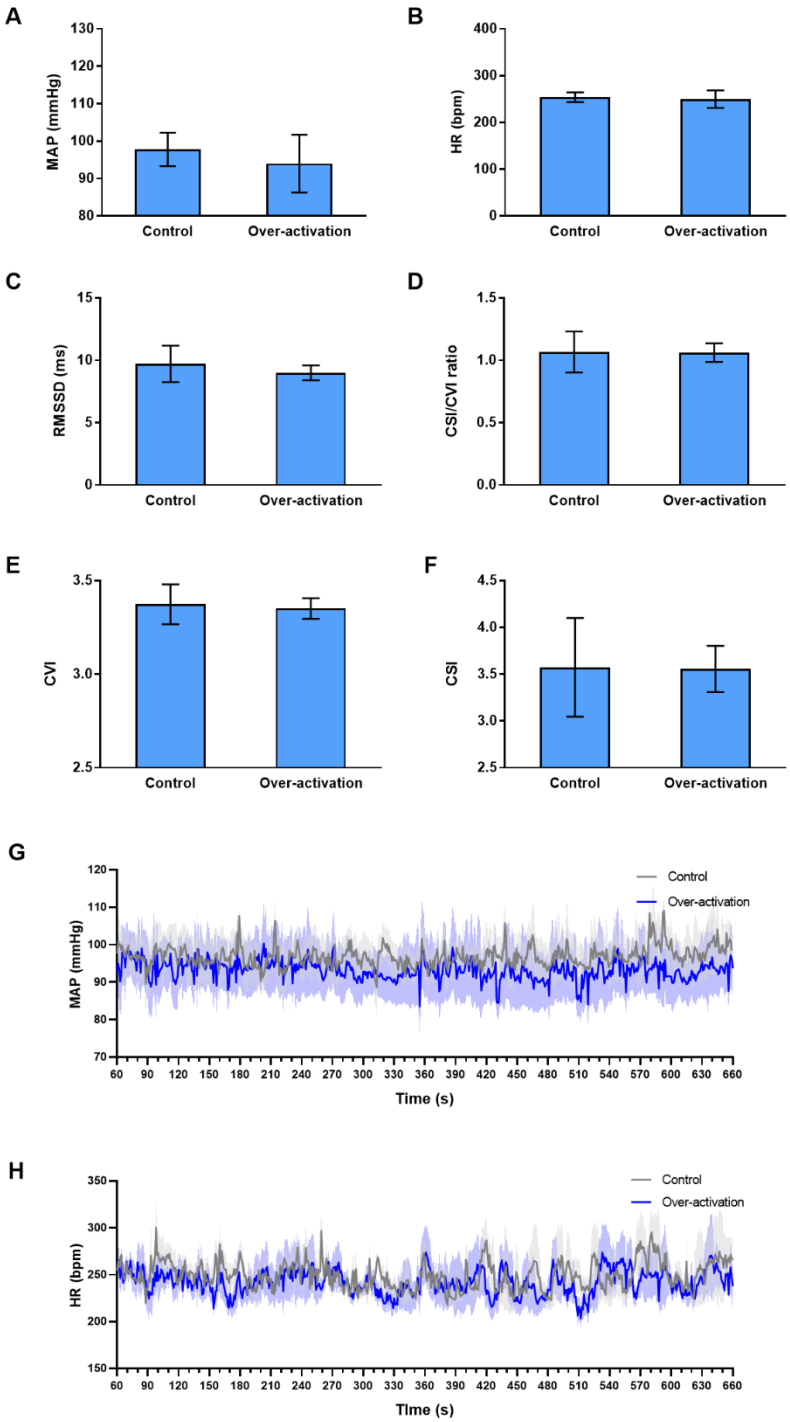


Figure 3-4 PgACC/32 over-activation had no effect on baseline cardiovascular function.
Relevant graphs show mean ± SEM. P values for **A-F** reported from two-tailed paired *t*-tests. N=5. PgACC/32 over-activation had no effect on **A** baseline MAP (p=0.604), **B** baseline HR (p=0.797), **C** HRV as measured by the RMSSD of successive IBIs (p=0.567), **D** sympathetic:parasympathetic balance as measured by CSI:CVI ratio (p=0.964), **E** CVI (p=0.780) or **F** CSI (p=0.960). **G** From second-by-second MAP values plotted across the entire analysis window, whilst there was a time dependent effect of pgACC/32 over-activation, it had no effect to systematically change MAP (manipulation × time, $F_{1,5560}$=117, p<0.0001; effect of manipulation, p=0.911). **H** From second-by-

second HR values plotted across the entire analysis window whilst there was a time dependent effect of pgACC/32 over-activation, it had no effect to systematically change HR (manipulation × time, $F_{1,5561}$=4.02, p=0.045; effect of manipulation, p=0.652).

3.4.5 Both sgACC/25 and pgACC/32 over-activation have no effect on baseline salivary cortisol levels

In three animals, we collected salivary cortisol samples before ('pre') and after ('post') saline control and over-activation manipulations of sgACC/25 and pgACC/32. Neither sgACC/25 nor pgACC/32 over-activation had any effect on baseline salivary cortisol levels as measured by the ratio of 'post':'pre' salivary cortisol levels (**FIGURE 3-5A, B**). However, it is important to note that there was substantial variability in the 'post':'pre' ratios for the control infusions into pgACC/32 (**FIGURE 3-5B**) which may have obscured any differences. Further work is needed to more comprehensively clarify the effects of pgACC/32 manipulations on cortisol levels under baseline conditions.

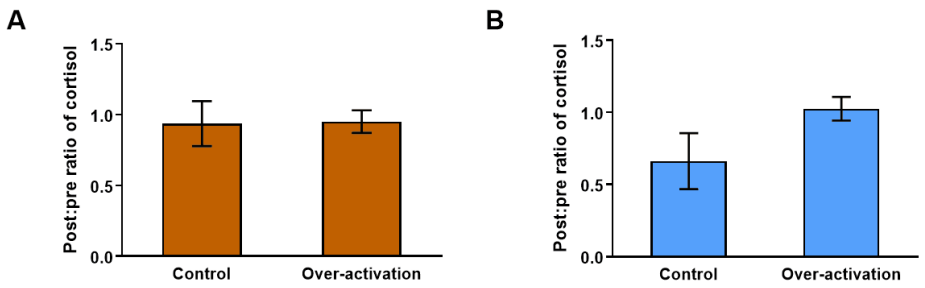


Figure 3-5 Neither sgACC/25 nor pgACC/32 over-activation has an effect on baseline salivary cortisol levels. Relevant graphs show mean ± SEM. N=3. **A** SgACC/25 over-activation had no effect on the ratio of 'post':'pre' cortisol ratios (two-tailed paired *t*-test, p=0.960). Neither control nor over-activation manipulations significantly altered the post:pre ratio from a value of 1.0 (indicating no change; one sample paired *t*-test, p=0.731 for control and p=0.603 for over-activation). **B** PgACC/32 over-activation had no effect on the ratio of 'post':'pre' cortisol ratios (two-tailed paired *t*-test, p=0.281). Neither control nor over-activation manipulations of sgACC/25 significantly altered the 'post':'pre' ratio from a value of 1.0 (indicating no change; one sample paired *t*-test, p=0.223 for control and p=0.785 for over-activation).

3.5 DISCUSSION

In this chapter, the effects of sgACC/25 and pgACC/32 over-activation were assessed in a relatively emotionally neutral condition (a familiar environment in which the animals had been habituated). The data presented here show for the first time that sgACC/25 over-activation is causally related to altered cardiovascular function but has no effect on outputs of the HPA axis under baseline conditions. PgACC/32 over-activation appears to have no effect on either cardiovascular or HPA axis function.

3.5.1 'At rest' cardiovascular function is profoundly altered by sgACC/25 over-activation

Over-activity in sgACC/25 has been implicated with enhanced low mood, depression (Drevets et al., 2008b; Harrison et al., 2009; Mayberg et al., 2005; Phan et al., 2002) and anxiety (Krain et al., 2008). These disorders are typified by enhanced negative emotion but are associated with altered cardiovascular function – particularly a reduction in HRV (Brunoni et al., 2013; Chalmers et al., 2014; Stapelberg et al., 2012; Wang et al., 2013). Understanding the mechanistic basis of the relationship between psychiatric disease and peripheral cardiovascular change is crucially important for several reasons:

- **Mood disorders and cardiovascular disease are frequently co-morbid**. Over 40% of patients with acute coronary syndrome have significant depressive symptoms (Carney et al., 2001) which frequently persist after discharge from hospital (Bush et al., 2005).
- **Mood and anxiety disorders are associated with an increased risk of cardiovascular disease**. Bulk data from prospective studies which include measures of depression symptom severity together with outcome measures from cardiovascular disease are supportive of depression as an important risk factor (Davidson, 2012; Frasure-Smith and Lespérance, 2005). Several studies have also found support for a link between anxiety disorders and the development of coronary heart disease (Vogelzangs et al., 2010).
- **Depressive mood changes are significant negative prognostic indicators in patients with cardiovascular disease**. A meta-analysis by van Melle and colleagues found that post-myocardial infarction (MI) depression is associated with a 2-2.5x increased risk of poorer cardiovascular outcome, together with an increased risk of new cardiovascular events (van Melle et al., 2004).

Subregions of the vmPFC have been implicated in cardiovascular regulation in rodents (Loewy and Spyer, 1990), NHPs (Wallis et al., 2017) and humans (Beissner et al., 2013). The combined implication of these subregions in both mood disorders and cardiovascular

regulation renders them a natural target for investigation into the causal basis of physiological dysfunction associated with psychiatric disease. However, the causal consequences of over-activity in NHP vmPFC subregions such as sgACC/25 and pgACC/32 on autonomic function have never been investigated. Here we demonstrate for the first time that sgACC/25 over-activity is causally related to cardiac changes characteristic of disorders associated with enhanced negative emotion.

Whilst not affecting baseline MAP, sgACC/25 over-activation significantly increased resting HR and reduced HRV as indexed by a reduction in the RMSSD of successive IBIs. Analysis of the geometric characteristics of the Poincare plot of IBIs meant that changes in cardiovascular indices could be fractionated into changes in vagal tone (CVI) *vs.* changes in sympathetic output (CSI) according to methods described in (Toichi et al., 1997). This analysis revealed that the cardiovascular effects of sgACC/25 over-activation appeared to be mediated predominantly by a reduction in CVI, although there was a trend effect to increase CSI.

The subgenual portion of the vmPFC (including sgACC/25 and BA10) has been related to vagal reactivity previously: variation in the high-frequency component of HRV (thought to reflect mainly parasympathetic tone) is strongly correlated with sgACC BOLD signal (Allen et al., 2015; Lane et al., 2013). How changes in BOLD signal relate to sgACC/25 *output* remains unclear – nevertheless, these studies do support a correlative link between sgACC/25 activity and vagal reactivity. Here, we have shown that this relationship may be causal. A predominant influence of sgACC/25 on parasympathetic – rather than sympathetic – branches of the ANS would also explain why over-activation is associated with an increase in HR (under predominant parasympathetic control through vagal innervation of the sinoatrial node), without an effect on MAP (predominant sympathetic control through vasomotor actions at the arteriolar level) (Thomas, 2011) in ‘at rest’ conditions.

Given the causal relationship between reduced vagal tone and sgACC/25 function demonstrated herein, one might speculate that VNS – a novel treatment for depression – may exert some of its therapeutic effects through modulation of sgACC/25. Indeed, chronic stimulation of the vagal nerve reduces subgenual prefrontal metabolism with the earliest changes detectable in sgACC/25 itself, extending rostrally to encompass the whole of sgACC (including BA10/14) over a 6-12 month period (Nahas et al., 2007; Pardo et al., 2008). The efficacy of VNS remains to be determined, although chronic stimulation seems to be particularly beneficial in reducing symptom severity (Sackeim et al., 2001; Schlaepfer et al., 2008). Further experiments could explore (i) the effects of stimulating the vagal nerve on metabolic activity within marmoset sgACC/25; (ii) whether ligating the vagus nerve mimics

the effects of sgACC/25 over-activity in the common marmoset; or (ii) whether lesions of sgACC/25 result in chronic changes in parasympathetic nervous function.

The lack of effect of pgACC/32 over-activity on baseline cardiovascular function suggests that dysfunctional activity within this region associated with mood disorders is not causally related to tonic alterations in autonomic function. However, activity and connectivity of a perigenual region including pgACC/32 has been associated with cardiovascular responses during physical exertion and emotional stressors (Gianaros and Wager, 2015; Gianaros et al., 2007; Ryan et al., 2011). Therefore, whilst over-activity in pgACC/32 may not be related to 'at rest' cardiovascular changes, it may be important in cardiovascular responses during physical or emotional stress and in situations of task-dependent modulation.

3.5.2 Endocrine function

The results reported here indicate that neither sgACC/25 nor pgACC/32 over-activation is causally related to baseline increases in levels of cortisol. This is consistent with rodent work, showing that lesions targeting IL and PL do not alter baseline cortisol levels (Diorio et al., 1993). Whilst HPA axis dysregulation is widely reported in depressed patients (Keller et al., 2017) and patients with anxiety (Faravelli et al., 2012), whether these patients tonically hyper-secrete cortisol under baseline conditions remains an issue of contention. Some estimates place the fraction of depressed patients hyper-secreting cortisol as high as 50% (Cowen, 2002) although the rate generally depends on the population of patients being sampled and is, in some cases, much lower (Cowen, 2002; Maes et al., 1993; Strickland et al., 2002). Furthermore, elevated cortisol secretion does not appear to be specific to depression – individuals dealing with chronic difficulties (for example, caring for relatives with dementia) can show increased cortisol secretion without necessarily having a mood disorder (Da Roza Davis and Cowen, 2001). Therefore, whether elevated basal cortisol levels should be expected from a manipulation that induces other translationally-appropriate physiological changes is unclear. The effects of over-activity in primate sgACC/25 on cortisol dynamics during stress are further explored in **Chapter 5**.

3.6 Conclusion

The results presented here demonstrate the causal contributions of over-activity in sgACC/25 to cardiovascular dysfunction associated with mood and anxiety disorders. The link between cardiovascular disease and elevated cardiovascular mortality associated with such psychiatric conditions may be mediated – at least in part – by sgACC/25 over-activity induced reductions in vagal tone. Targeted treatments aimed at modulating sgACC/25 over-activity (such as DBS) may therefore have beneficial effects on peripheral cardiovascular function; and conversely, treatments modulating parasympathetic tone (such as VNS therapy) may have central effects mediated through sgACC/25. The intimate relationship

between subjective and physiological states means that treatments modulating either of these domains will invariably have an impact on the other, with changes in both potentially contributing to therapeutic efficacy.

4 FRACTIONATED ANHEDONIA INDUCED BY OVER-ACTIVATING PRIMATE SGACC/25

A version of this chapter has been accepted for publication in the journal *Neuron*.

Abbreviation	Meaning
^{18}F-FDG PET	^{18}Fluorine-fluorodeoxyglucose positron emission tomography
5HT	Serotonin
ANOVA	Analysis of variance
BrkP	Breakpoint
CGP/LY	CGP52432/ LY341495
CPAS	Chapman Physical Anhedonia Scale
CS	Conditioned stimulus
DAB	Diaminobenzidine
dACC	Dorsal anterior cingulate cortex
DHK	Dihydrokainic acid
dmPFC	Dorsomedial prefrontal cortex
DSM	Diagnostic and Statistical Manual of Mental Disorders
EAAT2	Excitatory amino acid transporter-2
FCPS	Fawcett-Clark Pleasure Scale
FR	Fixed ratio
GABA	*γ*-aminobutyric acid
HR	Heart rate
IL	Infralimbic (cortex)
MAP	Mean arterial pressure
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex
MRF	Medullary reticular formation
MRI	Magnetic resonance imaging
NHP	Non-human primate
NMDA	*N*-methyl-*D*-aspartate (receptor)
NS	Not significant
NST	Nucleus of the solitary tract
OA	Over-activation
PFC	Prefrontal cortex
pgACC	Perigenual anterior cingulate cortex
PL	Prelimbic (cortex)
SEM	Standard error of the mean
sgACC	Subgenual anterior cingulate cortex
SpO_2	Oxygen saturations
SSRI	Selective serotonin reuptake inhibitor
SUVR(c)	Standard uptake value ratio (normalised to cerebellum)
US	Unconditioned stimulus
vmPFC	Ventromedial prefrontal cortex

4.1 Abstract

Anhedonia is a core symptom of depression, but its neurobiological mechanisms remain unknown. Correlative neuroimaging studies implicate dysfunction within the vmPFC, but the causal role of specific subregions has not been investigated. We addressed these issues by combining intracerebral microinfusions with cardiovascular and behavioural monitoring in marmoset monkeys to show that over-activation of NHP sgACC/25 causes anticipatory but not consummatory anhedonia, whereas manipulations of adjacent pgACC/32 have no effect. We further show that sgACC/25 over-activation induces motivational anhedonia. ^{18}F-FDG PET imaging reveals over-activation induced metabolic changes in a circuit involved in reward processing and interoception. Treatment with ketamine ameliorates anticipatory anhedonia and reverses associated metabolic changes, highlighting its utility in reward-related dysfunction. These results demonstrate a causal role for primate sgACC/25 over-activity in selective aspects of anhedonia, and ketamine's modulation of an affective network to exert its action.

4.2 Introduction

MDD is a common and debilitating condition which contributes significantly to global disease burden (Ferrari et al., 2013). Anhedonia – defined as a loss of interest or pleasure in all or almost all activities – is a core feature of MDD as outlined by the DSM-V (American Psychiatric Association, 2013). The clinical importance of anhedonia is illustrated by its high prevalence (Kessler et al., 2009; Pelizza and Ferrari, 2009) and its robustness as a negative prognostic indicator (Fawcett et al., 1990; McMakin et al., 2012; Spijker et al., 2001; Uher et al., 2012). Despite this, anhedonia remains poorly characterized both psychologically and neurobiologically.

Psychologically, the majority of studies fail to recognize its distinct behavioural subtypes, including anticipatory, motivational and consummatory components (Der-Avakian and Markou, 2012; Treadway and Zald, 2011). Instead, clinical and preclinical measures of anhedonia are almost exclusively consummatory. Clinical studies use scales to measure anhedonia such as the FCPS (Fawcett et al., 1983) and the CPAS (Chapman et al., 1976), in which the items are primarily concerned with the hedonic (consummatory) responses to reward. Similarly, rodent studies typically measure sucrose consumption as an overall index of anhedonia (Slattery et al., 2007; Tye et al., 2013). However, there is a fundamental disconnect between the construct assessed in these studies and the pattern of impairments manifested in depressed patients, who typically display anhedonic symptoms in anticipatory and motivational domains (Klein, 1987; Treadway and Zald, 2011) with intact consummatory responses (Amsterdam et al., 1987; Berlin et al., 1998; Dichter et al., 2010). It is also important to recognise that anhedonia is linked to apathy – sometimes termed amotivation –

and that apathy likely represents the instrumental, motivational components of anhedonia generally thought of as a problem with behavioural activation (especially in the context of neurological disorders such as Parkinson's disease) (Husain and Roiser, 2018).

Neurobiologically, whilst correlative human neuroimaging studies have implicated subregions of the vmPFC in the aetiology of depression, the precise anatomical locus of these changes varies throughout the sgACC/pgACC. In depressed subjects, over-activity in sgACC (including sgACC/25) has been reported (Drevets et al., 2008a; Keedwell et al., 2009; Mayberg et al., 2005), together with increased resting-state functional connectivity of this region to the default-mode network (Greicius et al., 2007). In neighbouring pgACC (including pgACC/32) there are variable reports of under-activity (Fitzgerald et al., 2008; Ito et al., 1996; Mayberg et al., 1994) and over-activity (Drevets et al., 1992; Ebert and Ebmeier, 1996; Ebert et al., 1994). Comparatively few studies have assessed the involvement of these regions in anhedonia specifically. Those that have link anhedonia in depressed patients (Keedwell et al., 2005) and trait anhedonia in healthy controls (Harvey et al., 2007) to over-activity in a perigenual region encompassing pgACC/32. Crucially, whether these changes are causal or compensatory remains unknown and this question cannot be answered with neuroimaging alone. Whilst interventional studies in rodents have attempted to address the issue of causality, progress has been hampered and translation made difficult owing to (i) a lack of functional equivalence between rodent vmPFC and human vmPFC (Myers-Schulz and Koenigs, 2012; Wallis et al., 2017), (ii) a failure to differentiate between the functionally distinct PL and IL vmPFC sectors in the rodent (for example, Ferenczi et al., 2016) and (iii) a lack of validity of the rodent sucrose consumption test as a measure of anhedonia relevant to depression (Dwyer, 2012). The issue is therefore best addressed by using interventional studies in non-human primates in which the anatomical organization of the vmPFC most closely resembles that of humans, and by recognizing the distinct subtypes of anhedonia.

The present study determines whether over-activity in sgACC/25 and under-/over-activity in pgACC/32 reported in depressed humans can cause anhedonia in marmosets. To establish causality, we pharmacologically manipulated these regions and assessed the impact on both autonomic (cardiovascular) and behavioural arousal. Utilizing two separate measures allowed us to bridge an additional translational gap associated with the assessment of emotion: rodent studies typically assess behaviour, whilst human studies often measure physiology and subjective responses using questionnaires. To maximize translational potential and to fully characterize any resultant anhedonia, we determined the impact of manipulations on (i) an appetitive Pavlovian conditioning task measuring reward anticipation (during the CS) and reward consumption (during the US) independently; (ii) an instrumental progressive ratio task measuring reward motivation; and (iii) the sucrose preference test

measuring reward consumption analogous to the assessment used in rodents. Our hypothesis was that an experimentally-induced anhedonia possessing face validity to the clinical state would lead to diminished reward anticipation and motivation, but not reward consumption.

Although clearly important, the vmPFC and its subregions do not operate in isolation. SgACC/25 and pgACC/32 function within a wider network of cortical and subcortical areas involved in the regulation of cognition, behaviour and physiology (Etkin et al., 2011). A comprehensive account of the role of vmPFC subregions in anhedonia necessitates investigation of their interaction with downstream structures, which may contribute to the behavioural and physiological phenotypes observed following their manipulation. By combining intracerebral infusions with ^{18}F-FDG PET imaging, we sought to determine the network of brain regions associated with specific symptoms of anhedonia.

An appreciation of the neurobiological substrates underlying distinct components of anhedonia will facilitate the development and evaluation of novel treatments. Ketamine has recently emerged as a promising glutamate-based antidepressant, demonstrating efficacy in treating reward-processing deficits which are otherwise resistant to conventional medication such as SSRIs (Argyropoulos and Nutt, 2013; Lally et al., 2014, 2015; Nutt et al., 2007; Parsaik et al., 2015). Whilst sub-anaesthetic doses of ketamine have been found to reverse consummatory anhedonia in rodent models of depression (Garcia et al., 2009; Li et al., 2011), the effect of ketamine on highly relevant components of anhedonia has not been investigated. Furthermore, the neural mechanisms by which ketamine exerts its efficacious action remain unclear. Consequently, we determined the efficacy of ketamine to alleviate anhedonic deficits and associated circuit-wide changes induced by selective manipulations of marmoset vmPFC. In doing so, we aimed to provide novel insight into the neurobiological basis of ketamine's action.

4.3 METHODS

4.3.1 Subjects

Nine marmosets (seven females, two males) took part in this study. These marmosets were Subjects 8-16 of cohort two, described in **2.1.1 SUBJECTS**. The marmosets were housed and cared for as described in **2.1.2 HOUSING**.

4.3.2 Surgical procedures

Nine animals underwent two aseptic surgical procedures: one to implant intracerebral cannulae targeting either sgACC/25 alone or both sgACC/25 and pgACC/32, and one to implant a telemetric blood-pressure monitor into the descending aorta. Four of these animals underwent a third procedure to implant a vascular access port for administration of radioactive ligands (see **2.2.5 SOLOPORT SURGERY**).

4.3.3 Behavioural testing apparatus and paradigms

4.3.3.1 Appetitive discriminative conditioning

Behavioural testing took place within a sound-attenuated box in a dark room. The test chamber was lit by a 3W bulb (houselight), located in the middle of the ceiling of the chamber. A removeable module consisting of two electrically controlled food-box units was attached to the left and right walls of the internal frame of the apparatus. A telemetry receiver was concealed beneath the floor of the apparatus. Each food-box was cylindrical (internal diameter 52mm and length 51mm). When the carry box was fitted into the internal frame of the apparatus, the positions of the windows were aligned with the food-boxes. The inside of each food-box could be illuminated by a 28V, 0.04W encased light bulb. Access to both food-boxes was restricted by black and opaque Perspex doors, which could be opened remotely to allow access. The chamber contained computer-controlled speakers through which auditory stimuli could be played, and three cameras used to record the animal during testing using video software (CyberLink, Power Director, CyberLink Corp.). The video display was shown on a monitor outside of the testing apparatus meaning the animal could be observed by the experimenter during testing. The apparatus was controlled by the Whisker control system (Cardinal and Aitken, 2010) and in-house software.

Prior to conditioning, all marmosets were habituated to the sight and sound of the food-box doors opening and closing. During these sessions, high incentive food (several pieces of marshmallow) was presented in either the left or right food-box and the door of the food-box was opened after 120s. When the animal stopped showing a startle response (i.e. rearing and jumping) to the opening of the door and started consuming marshmallow within 40s, they were advanced to conditioning sessions. The mean number of habituation sessions was 10 ± 1 (mean ± SEM).

Marmosets were then exposed to two novel auditory cues and the cardiovascular arousal response (MAP) was measured. The cue that produced the smallest arousal response became the CS+ and the cue that produced the largest arousal response became the CS-. The animals were then trained on an appetitive Pavlovian conditioning paradigm: the CS+ was associated with food reward (US+; marshmallow, net weight approximately 5.8-6.2g) and the CS- was associated with no reward (US-). A trial consisted of a 20s CS period during which one of the cues was played. At the end of this period, one of the food-boxes opened, accompanied by the houselight offset, the onset of the food-box light and presentation of either an empty box (US-) or the high-incentive food reward (US+). The auditory CS continued to be played for the entire 120s duration of the US period. In multiple-trial sessions, the offset of the US period was indicated by termination of the CS, closure of the black opaque food-box door and onset of the houselight. If the trial was the last in a session, all lights were turned off at the end of the US period indicating session termination. The intervals between trials were pseudorandomly varied between 70-110s. There were either one or two trials in each session with no more than one CS+/US+ trial; if present, the CS+/US+ trial was always the final trial. Thus, a session could consist of a single CS-/US- or CS+/US+ trial, two CS-/US- trials or one CS-/US- trial and one CS+/US+ trial (see **TABLE 4-1** for testing schedule).

Day	Session	ITI (s)	
Mon	CS-/CS-	110, 70	
Tue	CS+	70	
Wed	CS-/CS+	100,80	**(± saline infusion)**
Thurs	CS-	90	
Fri	CS-/CS+	70, 110	**(± drug infusion)**
Mon	CS-	100	
Tue	CS-/CS-	100, 80	
Wed	CS-/CS+	110,70	**(± saline infusion)**
Thurs	CS-	80	
Fri	CS-/CS+	80, 100	**(± drug infusion)**

Table 4-1 Schedule for training on the Appetitive Discrimination paradigm. No more than five sessions containing a CS+ were given over a 10-day period. Infusions of saline or drug were carried out on CS-/CS+ sessions. ITIs were pseudorandomly varied across sessions. The mean ITI was 90s.

Infusions were always conducted on sessions containing both CS-/US- and CS+/US+ trial types which lasted 460s in total. Marshmallows were chosen as the food reward since marmosets invariably favour them over other types of food (Caldwell et al., 2009). Behavioural and cardiovascular measurements were taken both during the CS and US periods as well as the 20s baseline periods immediately prior to the onset of the CS.

Marmosets undergoing ^{18}F-FDG PET scanning were trained on a modified version of the Pavlovian conditioning paradigm. The length of the session was increased from 460s to 1800s to facilitate adequate ligand uptake during testing. Marmosets were habituated to the increased length of the session by gradually increasing the time spent in the testing apparatus over approximately 12 habituation sessions (12 ± 1.4, mean ± SEM). At 600s and 1200s, the opaque door of the rewarded food-box opened for 20s revealing the high-incentive food reward, after which it closed again. During this period, marmosets could see the reward without being able to access it – the sight of reward is also known to act as an appetitive CS (Braesicke et al., 2005). At 1660s, the CS+ auditory cue was played for 20s after which the rewarded food-box opened for 120s as before (US+). The CS+ continued to be played for the entire 120s of the US+ period. Marmosets received at least 5 of these sessions before undergoing the first PET scan. Owing to the requirement for anaesthesia during PET scanning, marmosets were unable to consume food reward on the day of the scan. Therefore, in sessions conducted on the day of scanning, animals were removed from the apparatus at 1680s (immediately after experiencing the CS+) and the session was terminated without a US+.

4.3.3.2 Progressive ratio

Behavioural testing took place within a sound-attenuated box in a dark room, in a chamber similar to that described above with foodbox units replaced with a touchscreen (Campden Instruments, Loughborough, UK) and milkshake spout. When the carry box was fitted into the internal frame of the apparatus and the door removed, the marmosets had access to the touchscreen. The stimulus presented on the screen was a white circle (300 pixels in diameter) displayed to the left or right of the central spout via the Whisker control system. When appropriate, a reward of cooled banana milkshake (Nestlé) was delivered through a centrally placed spout for 5s. A brief tone (0.5s, 80dB) played from speakers at the back of the testing chamber signalled reward availability.

Marmosets were first familiarized with the delivery of banana milkshake from the spout. They were then trained to respond to stimuli presented on a touchscreen for reward. Once marmosets were reliably and accurately making $\geq$30 responses in 10 minutes to a green square presented to the left or right of the licker (see (Roberts et al., 1988)), the stimulus was changed to a white circle presented at a fixed location (the animal's preferred side). Fixed

ratio (FR) response schedules were then introduced to familiarize marmosets with the requirement to make repeated responses for reward. Marmosets progressed from FR1 → FR2 → FR3 → FR5 → FR7 response schedules when their performance at each level was stable. After FR7, marmosets were trained on a progressive ratio schedule of reinforcement taken from (Pryce et al., 2004). In this schedule, the response increment from trial *n* to *n*+1 starts at +1 and then doubles every eight trials until a maximum increment of 8 (+1 → +2 → +4 → +8 until end). After two minutes of inactivity (or a session length of 30 minutes), marmosets 'timed-out' and were removed from the apparatus. The total number of responses marmosets made prior to timing-out was considered the breakpoint.

4.3.3.3 Sucrose preference test

The sucrose preference test was carried out in animals' home cages (see **2.3.3 HOME CAGE**). During a testing session, animals were divided into the top left or top right quadrant of the cage and the nest-box was removed.

Marmosets were presented with two bottles identical in appearance: one water bottle and one containing sucrose (25g in 250g water). This concentration of sucrose was decided upon based on pilot experiments showing that it was the minimum concentration needed to obtain reliable preferences of over 90%. Each session lasted two hours, and from session to session the left-right position of the two bottles was varied. Every 30 min, an experimenter briefly removed each bottle and weighed it, before replacing it in the same position. The amount of sucrose consumed and sucrose preference (sucrose/[sucrose+water]) was measured over the session. Once marmosets achieved stable sucrose preference $\geq$90% over two sessions, experimental manipulations took place. The number of sessions required to obtain this was 4.5 ± 0.6 (mean ± SEM).

4.3.4 Drug Treatments

Central and peripheral drug treatments were carried out as described in **2.4 DRUG TREATMENTS**. The pharmacological compounds used in experimental manipulations in this study were: 0.9% saline (vehicle control), DHK (an EAAT2 inhibitor), CGP52432/ LY341495 (a combination of a $GABA_B$ and $mGlu_{2/3}$ receptor antagonist), muscimol/baclofen (a combination of a $GABA_A$ and $GABA_B$ receptor agonist), citalopram (an SSRI), naloxone (a μ-opioid receptor antagonist) and ketamine (an NMDA receptor antagonist). For details of doses and pre-treatment times, see **TABLE 2-4**.

4.3.5 PET imaging

Each animal selected to undergo PET scanning received three ^{18}F-FDG PET scans with a microPET Focus-220 scanner (Concorde Microsystems, Knoxville, TN) with the first scan approximately 2 weeks after port implant surgery, and the interval between scans

approximately 2 weeks. On the day of a scan, animals received no breakfast to lower blood glucose concentration and hence increase the transport of ^{18}F-FDG into brain tissue, thereby increasing the cerebral ^{18}F-FDG signal-to-noise ratio. The animal received an infusion of either saline vehicle or DHK approximately 10 minutes prior to a bolus injection of approximately 70MBq of ^{18}F-FDG administered subcutaneously via the vascular access port. After 30 minutes of the behavioural paradigm described in **4.3.3.1**, the animal was anaesthetised. The animal was then placed on a heat-pad on the scanner bed and attached to monitoring equipment. Heart rate, SpO_2 and respiration was monitored continuously. The bed of the scanner was positioned to locate the brain in the centre of the PET scanner field-of-view, where both sensitivity and resolution are optimal. For consistency, PET data acquisition started 70 minutes after the ^{18}F-FDG injection and lasted for 45 minutes. The energy and coincidence timing windows used were 350-650keV and 6 nanoseconds, respectively.

The listed mode data were histogrammed into 9 x 5 minute 4D sinograms and then reconstructed using Fourier rebinning (Defrise et al., 1997) followed by the 2D ordered subsets expectation maximization algorithm installed on the scanner (6 iterations, 16 subsets). As post-injection transmission scanning was not feasible, attenuation correction used a mean non-attenuation corrected ^{18}F-FDG image to determine a body outline, within which a uniform attenuation coefficient (0.096cm^{-1}) was ascribed. This was combined with a standard attenuation map of the carbon fibre bed determined from transmission scanning. The combined attenuation map was forward projected using software installed on the scanner to produce an attenuation correction factor sinogram, and image reconstruction was repeated with attenuation correction applied. Corrections were also applied from randoms, dead-time, normalization, sensitivity and decay.

4.3.6 Data acquisition and preliminary analysis

For studies involving telemetric measurements, MAP and H R values were collected as described in **2.6.1 TELEMETRY DATA COLLECTION AND ANALYSIS**. MAP is used as the principal cardiovascular measure for two reasons: firstly, in the appetitive discriminative conditioning paradigm, cardiovascular conditioning was less variable with MAP values compared to HR values (see **4.4.2**). Secondly, MAP was unaffected by DHK infusions in the neutral condition, whereas HR values are confounded by a baseline cardiovascular effect (**Chapter 3**).

4.3.6.1 Appetitive discriminative conditioning

A mean MAP and HR value was calculated over the 20s CS period. The immediate 20s preceding each CS period served as its baseline for comparison purposes: the CS directed autonomic measures were calculated as the difference between the mean value for CS

period and the mean value for the baseline period (e.g. $MAP_{CS} – MAP_{baseline}$). The principal measure for the consummatory period was the US directed MAP response, calculated as the difference in MAP response between the US period (calculated as a mean MAP response after the animals began consuming the reward) and 20s CS period ($MAP_{US} – MAP_{CS}$). MAP_{CS} was factored in to the response during the US in order to quantify MAP responses over-and above any response to the CS (which continued playing during the US period).

Behaviour during the discrimination was recorded and subsequently scored by an experimenter and a blinded research assistant. Behaviours were scored separately during the anticipatory period and consummatory period. The anticipatory (*i.e.* during the CS) behaviours scored were CS directed orienting behaviours known as 'head-jerks' (Reekie et al., 2008). The number of anticipatory head-jerks was compared to the value in the 20s preceding baseline period to give a CS directed score. The consummatory behaviour scored was the amount of reward consumed across the 120s period (g). Additionally, locomotion was scored as the total time an animal spent in motion (movement of all four limbs plus movement about the body axis) during the CS+ period (s). These were correlated against MAP changes to determine if reductions/increases in locomotion were associated with the observed autonomic changes.

4.3.6.2 Processing of PET data

MR imaging of the animals was not possible due to the cannulae implanted in the brain, preventing the use of MRI-based spatial normalization. Instead, first, the mean FDG image of each scan was manually, rigidly registered to an FDG brain template produced from another FDG study in the colony that included MRI. The FDG brain template was constructed by averaging *n*=21 FDG images transformed to template space using registration transformations obtained by warping MRI images (co-registered to the FDG images) to an MRI template. Secondly, for each subject, the three FDG scans rigidly registered to the FDG template were averaged, the resultant image was non-rigidly registered (affine and non-linear) to the FDG template using 'ANTS' (Avants et al., 2008), and this transformation was applied to each of the three rigidly registered FDG scans. Use of a single spatial normalization transformation per subject rather than per FDG scan was adopted after it was found – using the n=21 FDG scans with MRI – that this approach provided ROI PET values with a higher correlation to those obtained using MRI-based spatial normalization (R^2=0.89 vs. R^2=0.87). For each scan, a standard uptake value ratio (SUVR) map was created for voxel-wise analysis by dividing the mean PET image by a cerebellum ROI value (SUVRc). Normalization by the cerebellum signal was designed to minimize the confounding influence of inter-scan differences in tracer availability, plasma glucose concentration, the effect of anaesthesia on cerebral blood flow and metabolism, and basal cerebral glucose metabolism.

4.3.7 Statistical analysis

Where appropriate, data were inputted into GraphPad Prism v8.00.178 for Windows (GraphPad Software, La Jolla, CA) for statistical analysis. Significance was set at α=0.05 in all cases. In ANOVAs, multiple comparisons were corrected for using Sidak's multiple comparisons test unless planned comparisons were being made, in which case Fisher's LSD test was used.

4.3.7.1 Appetitive discriminative conditioning

To illustrate successful discrimination between CS+ and CS-, a two-tailed paired *t*-test was conducted on CS directed cardiovascular and behavioural measurements in sessions prior to drug manipulations. Cardiovascular discrimination between US+ and US- was assessed in the same way. CS measurements taken during drug manipulation sessions were compared to infusions of saline vehicle using a two-way repeated-measures ANOVA of the form $C_2 \times M_2$ where *C* is a within-subject factor with two levels (CS type: CS+, CS-) and *M* is a within-subject factor with two levels (manipulation type: saline, drug). Significant interactions were subjected to Sidak's multiple comparisons test applied to vehicle *vs.* drug data for CS+ and CS- (to ascertain whether there were changes in responses to the CS+ selectively, CS- selectively or both). US+ measurements taken during drug manipulations were compared to infusions of saline vehicle using a two-tailed paired *t*-test. In addition, CS directed changes in locomotion were correlated with CS directed MAP changes across saline, DHK and CGP52432/ LY341495 infusion sessions into sgACC/25. R^2 values were calculated to ascertain the strength of correlation between MAP change and locomotion change across infusion types.

For the ketamine study, cardiovascular and behavioural measurements were subjected to a two-way repeated-measures ANOVA of the form $C_2 \times T_3$ where *C* is a within-subject factor with two levels (CS type) and *T* represents timepoint with three levels (4 hours, 1 day, 7 days). Significant interactions were subjected to Sidak's multiple comparisons test, applied to vehicle *vs.* drug data across CS type. Ketamine control data were analysed using a two-way repeated-measures ANOVA of the form $C_2 \times M_2$ as described above. Cardiovascular and behavioural data from citalopram control and citalopram manipulation studies were analysed using two-way repeated-measures ANOVAs of the form $C_2 \times M_2$ as described above.

4.3.7.2 Progressive ratio

For control and drug sessions, a percentage change in the number of responses at breakpoint was calculated compared to the previous day. A two-tailed paired *t*-test was conducted on percentage change values for control *vs.* drug sessions.

4.3.7.3 Sucrose preference test

During drug manipulation sessions, a two-tailed paired *t*-test was conducted to compare preference values over the first 30-minute window in control *vs.* drug sessions. Sucrose and water consumption over the first 30-minute window were analysed using a two-way repeated-measures ANOVA of the form $M_2 \times S_2$ where *M* is a within-subject factor with two levels (manipulation type) and *S* is a within-subject factor with two levels (solution type). To compare the effect of drug manipulations on cumulative consumption of sucrose *vs.* cumulative consumption of water across the entire two-hour testing session, data were subjected to a three-way repeated-measures ANOVA of the form $M_2 \times S_2 \times T_4$ (*M*: within-subject factor of two levels [manipulation type]; *S*: within-subject factor of two levels [solution type]; *T*: within-subject factor of four levels [time window]). In the case of naloxone manipulations, planned comparisons were made between naloxone and control manipulations at each timepoint for water and sucrose solutions separately using Fisher's LSD test.

4.3.7.4 PET conditioning

A ratio was calculated for cardiovascular and behavioural measures during the CS+ for control scans *vs.* over-activation, and over-activation scans *vs.* over-activation with ketamine. A one sample *t*-test was performed to determine whether the ratio significantly differed from a hypothetical value of 1.0 (no difference).

4.3.7.5 PET scanning

SPM8 (Wellcome Trust Institute for Neurology, UCL, UK) was used for voxel-based analysis. A general linear model was configured with covariates for subject and condition (saline control vs. DHK vs. [DHK + Ketamine]) and changes in activity were tested with Student's t-test at each voxel. Prior to estimating the model, images were smoothed with a filter size of $1mm^3$ using a locally adapted Gaussian kernel to include only those voxels inside a brain mask. In mitigation against type I errors expected due to multiple comparisons, an adjusted p-value of $p<0.005$ was applied with an extent threshold adjusted for search volume of 26 voxels.

4.3.8 Post-mortem histological processing

4.3.8.1 Assessment of cannula placement

For all eleven animals, brain sections were prepared and visualized as described in **2.8 POST-MORTEM ASSESSMENT OF CANNULA PLACEMENT**.

4.3.8.2 Immunohistochemical assessment of cFos expression

One hour prior to perfusion, an animal was infused with DHK in the left sgACC/25 and saline vehicle contralaterally. The animal was euthanised, perfused and brains were sectioned

before the tissue was immunohistochemically processed for cFos expression to provide additional evidence that DHK is activating neurons within sgACC/25. It is important to note that this method alone does not distinguish between pyramidal neurons and interneurons. Sections were washed for 3 x 10 minutes in 0.01M PBS and incubated for 10 minutes in 10% methanol/10% H_2O_2 v/v solution to inhibit endogenous peroxidase activity. Sections were then washed and blocked for two hours with 3% normal goat serum before being incubated overnight with the primary antibody (1:2000 Rabbit polyclonal to cFos; ab190289, Abcam, Cambridge, UK). The following day, sections were washed and incubated for two hours with the secondary antibody (1:500 Goat Anti-Rabbit IgG H&L [Biotin]; ab6720, Abcam). After secondary incubation, sections were incubated in an avidin/biotin complex solution for 30 minutes (Vector Labs, Peterborough, UK) and then reacted in 3,3’ diaminobenzidine (DAB) chromogen for 15 seconds (ImmPact DAB SK-4105, Vector Labs). Following DAB reaction, sections were transferred to ice-cold PBS and mounted on gelatin-coated slides. Slides were dehydrated, cover-slipped using DPX mounting medium (Sigma-Aldrich, MI, US) and visualized using a Leitz DMRD microscope and the two hemispheres were compared qualitatively.

4.4 RESULTS

4.4.1 Post-mortem assessment of cannula placement and cFos expression

Histological assessment using cresyl violet staining confirmed that marmosets had cannula implanted successfully into sgACC/25 and/or pgACC/32 (**FIGURE 4-1A**). In one marmoset cFos expression was assessed following unilateral infusion of DHK (used to over-activate sgACC/25) into left sgACC/25, compared to a contralateral infusion of vehicle (saline) control. Throughout the rostro-caudal extent of sgACC/25, DHK elevated cFos expression levels compared to the contralateral side (**FIGURE 4-1B**). This serves as evidence demonstrating that infusions of DHK successfully increase activity of neurons within sgACC/25 (as measured by immediate early gene expression).

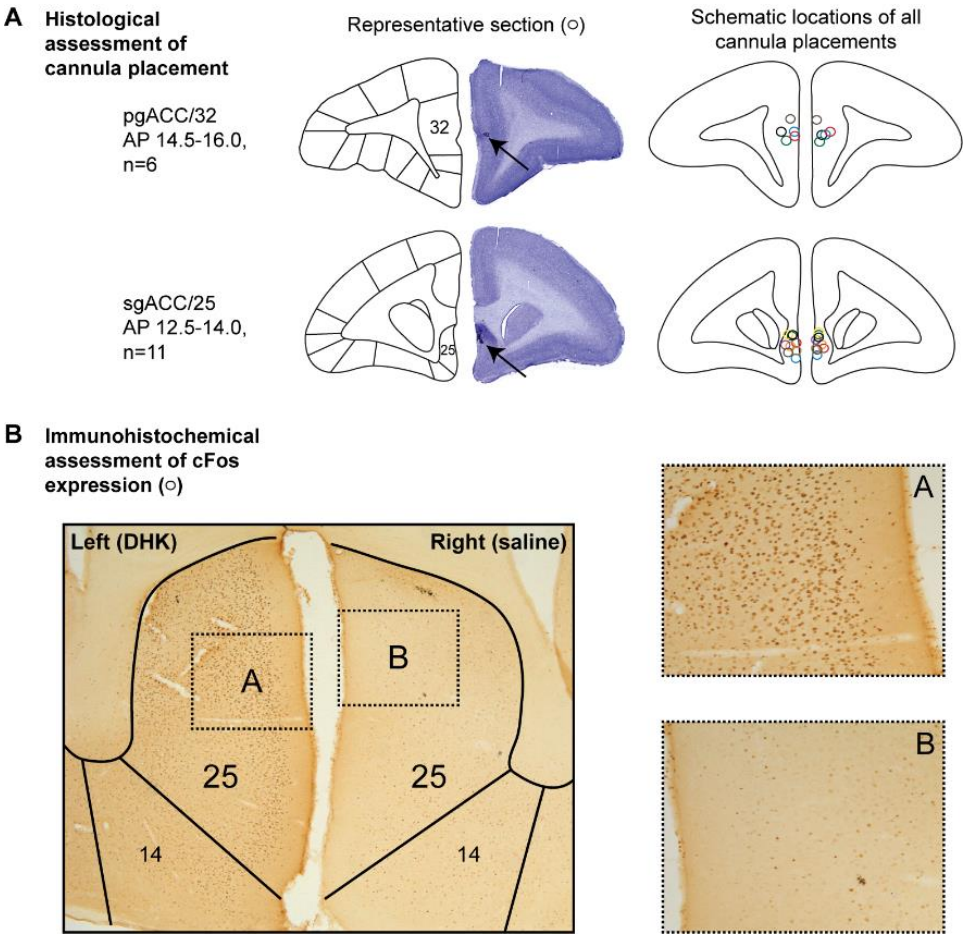


Figure 4-1 Cannula placements and cFos expression. **A** Histological assessment of cannula placement using cresyl violet staining. Representative sections are shown with pgACC/32 and sgACC/25 cannulation sites indicated. A schematic diagram shows the cannula placements for all monkeys reported in this chapter. **B** cFos expression was assessed in one marmoset following DHK infusion in left sgACC/25, and saline infusion in right sgACC/25. DHK infusions (inset A) – but not saline infusions (inset B) – caused robust cFos expression in sgACC/25.

4.4.2 SgACC/25 over-activation blunts anticipatory but not consummatory arousal for reward, whereas pgACC/32 manipulations have no effect

Following surgery and recovery (experimental outline shown in **FIGURE 4-2A**), marmosets (n=6) acquired appetitive Pavlovian discriminative cardiovascular and behavioural arousal responses to an auditory cue (CS+) predicting the presence of high-incentive food reward (US+), but not to a second auditory cue (CS-) predicting the absence of food reward (US-; **FIGURE 4-2B, C**). Successful discrimination was evident in cardiovascular responses as an increase in MAP during the CS+ (compared to the 20s-preceding baseline period) but not during the CS- (**FIGURE 4-2D**). During the US+ period in which the animals consumed the food reward, a rise in MAP was observed above the rise seen during the CS+ with no change during the US- (**FIGURE 4-2E**). Marmosets fail to show MAP rises when consuming non-preferred foods (Braesicke et al., 2005), suggesting that the increase observed during the US+ period was due to hedonic – rather than ingestive – factors. HR responses were variable: whilst there was a trend towards discrimination during the CS period (mean ± SEM difference between CS+ and CS-: 31 ± 14bpm, p=0.077), no discrimination was evident during the US period (mean ± SEM difference between US+ and US-: 2.9 ± 14bpm, NS). MAP is therefore used as the principal cardiovascular measurement throughout the study owing to its sensitivity as a discriminative measure of anticipatory and consummatory arousal.

Behaviourally, both discriminative conditioned CS directed and conditioned US directed behaviours were exhibited during the CS period. The principal CS directed behaviour was a rapid ‘head-jerk,’ previously described in rodents (Holland, 1977) and marmosets (Braesicke et al., 2005; Reekie et al., 2008) as an orienting response to an auditory appetitive CS. Animals developed increased head-jerking behaviour during the CS+ but not the CS- (**FIGURE 4-2F**). The US directed measure used was nose-poking towards the feeder box, but this was highly variable and did not discriminate between CS type (mean ± SEM difference between CS+ and CS-: 0 ± 1, NS). During the US+, the amount of food consumed was used as the principal behavioural index of reward consumption. The latency to begin eating food reward was also measured.

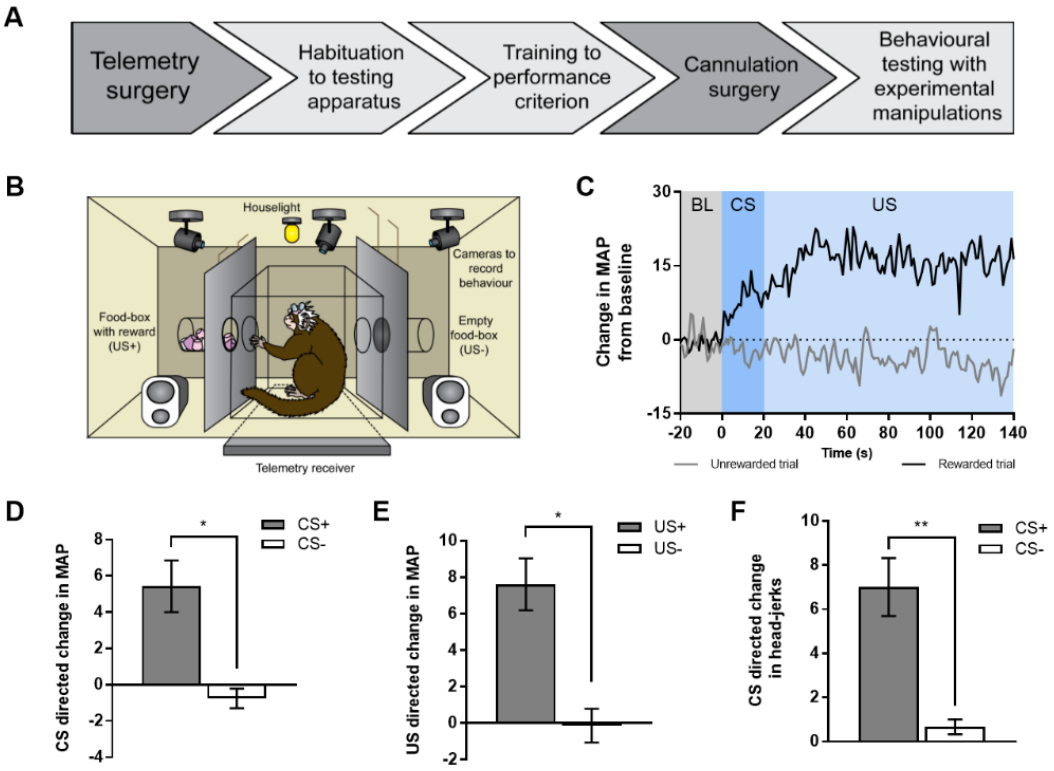


Figure 4-2 Experimental outline and conditioned discrimination. Relevant graphs show mean ± SEM for sessions immediately prior to experimental manipulations. N=6. **A** Experimental overview. Following telemetry surgery, marmosets were habituated to the testing apparatus for 5-10 sessions, trained on the appetitive discrimination task until criterion was reached (significant MAP discrimination over three CS+/CS- sessions, two-tailed paired *t*-test) and then cannulated to target sgACC/25 and pgACC/32. Following re-attainment of criterion post-surgery, experimental manipulations took place. **B** Diagram of conditioning apparatus. During discrimination sessions, two auditory cues predicted either the presence (CS+/US+) or absence (CS-/US-) of a high incentive food reward (marshmallow). A telemetry receiver placed underneath the apparatus recorded cardiovascular measurements which were sent to a computer in an adjacent room. **C** Example MAP trace during baseline ('BL,' 20s immediately prior to CS), CS (20s) and US (120s) periods for a rewarded and non-rewarded trial within a conditioning session. Values are calculated as a difference from the mean MAP during baseline. Animals showed an anticipatory MAP rise during the CS+ and a further consummatory rise during the US+. **D** Animals showed CS directed (CS minus baseline) anticipatory MAP responses to the CS+ but not the CS- (two-tailed paired *t*-test, p=0.013). **E** Animals showed US directed (US minus CS) consummatory MAP responses to the US+ but not the US- (two-tailed paired *t*-test, p=0.017). **F** Behaviourally, animals showed rapid orienting responses (head-jerks) to the CS+ but not the CS- (two tailed paired *t*-test, p=0.003).

To over-activate sgACC/25, marmosets received infusions of DHK to reduce glutamate reuptake (n=5) and/or CGP52432/ LY341495 (CGP/LY; $GABA_B$ receptor antagonist/$mGlu_{2/3}$ receptor antagonist) to increase pre-synaptic glutamate release (n=6). DHK-induced over-activation of sgACC/25 resulted in anticipatory but not consummatory anhedonia, selectively reducing MAP and behavioural responses during the anticipatory CS+ period (**FIGURE 4-3A, B**) but not during the consummatory US+ period (**FIGURE 4-3C, D**). CGP/LY over-activation of sgACC/25 also resulted in anticipatory but not consummatory anhedonia (matching the effect seen with DHK), by reducing anticipatory CS+ responses (**FIGURE 4-3E, F**) but not consummatory US+ responses (**FIGURE 4-3G, H**).

Neither manipulation caused a significant change in locomotor activity (**FIGURE 4-4A-C**), nor were there any changes in the latency to eat the food reward (**TABLE 4-2**).

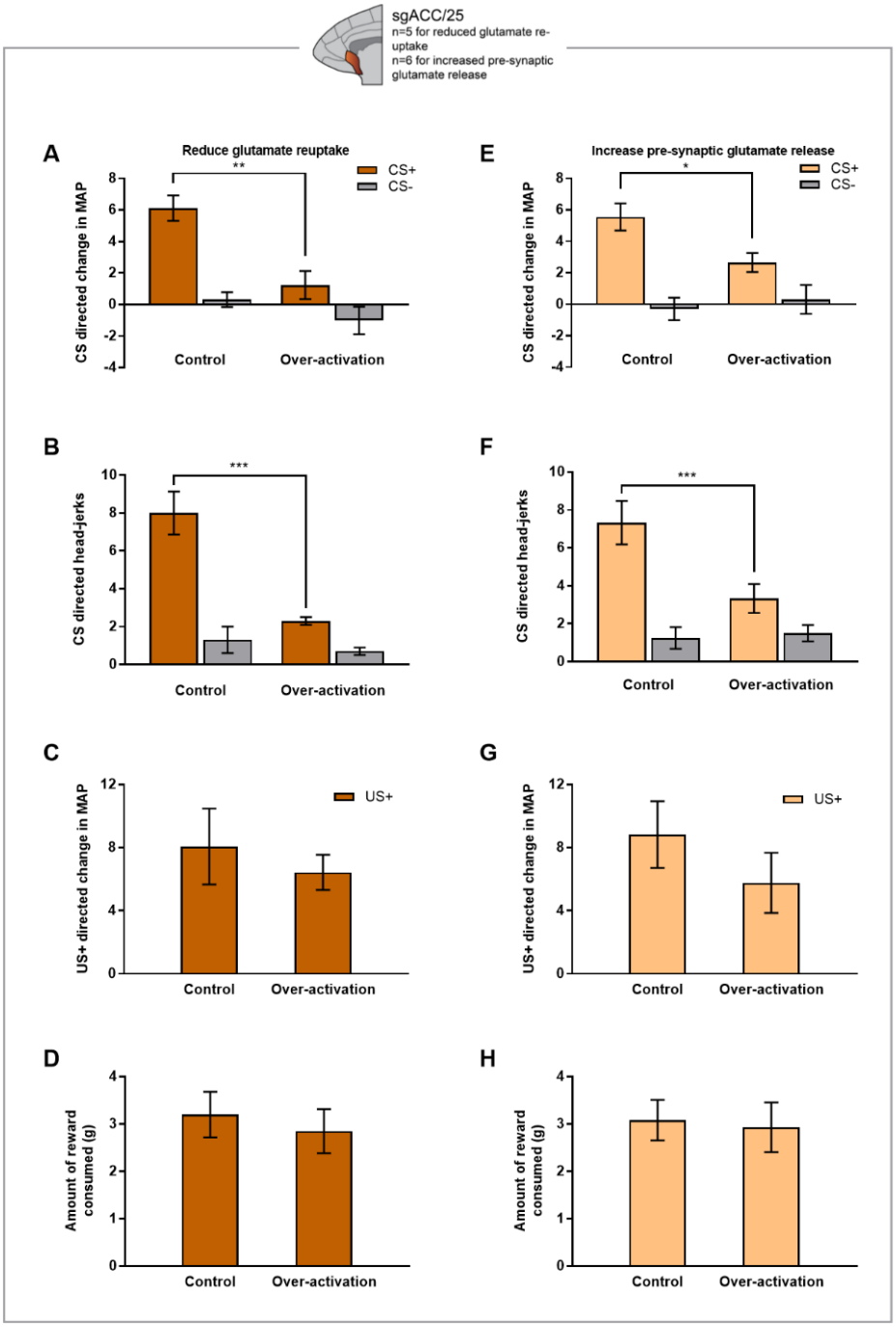


Figure 4-3 SgACC/25 over-activation impairs anticipatory responses but not consummatory responses. Relevant graphs show mean ± SEM. N=5 for reduced glutamate reuptake. N=6 for increased pre-synaptic glutamate release. **A** SgACC/25 over-activation by reducing glutamate reuptake (DHK) blunted anticipatory cardiovascular arousal in a CS-dependent manner (infusion × CS, $F_{1,4}$=10.63, p=0.031) decreasing responding to the CS+ but not the CS- (effect of infusion: CS+, p=0.006; CS-, p=0.301). **B** The same manipulation also blunted anticipatory behavioural arousal in a CS-dependent manner (infusion × CS, $F_{1,4}$=72.25, p=0.001), decreasing responding to the CS+ but not the CS- (effect of infusion: CS+, p<0.001; CS-, p=0.407). **C** There was no significant effect on consummatory cardiovascular arousal during the US+ (two-tailed paired *t*-test, p=0.451). **D**

There was no effect on reward consumption during the US+ (two-tailed paired *t*-test, p=0.241). **E** SgACC/25 over-activation by increasing pre-synaptic glutamate release (CGP/LY) also blunted anticipatory cardiovascular arousal in a CS-dependent manner (infusion × CS, $F_{1,5}$=14.39, p=0.013) decreasing responding to the CS+ but not the CS- (effect of infusion: CS+, p=0.014; CS-, p=0.634). **F** The same manipulation blunted anticipatory behavioural arousal in a CS-dependent manner (infusion × CS, $F_{1,5}$=48.08, p=0.001) decreasing responding to the CS+ but not the CS- (effect of infusion: CS+, p<0.001; CS-, p=0.839). **G** There was no significant effect on consummatory cardiovascular arousal during the US+ (two-tailed paired *t*-test, p=0.129). **H** There was no effect on reward consumption during the US+ (two-tailed paired *t*-test, p=0.665).

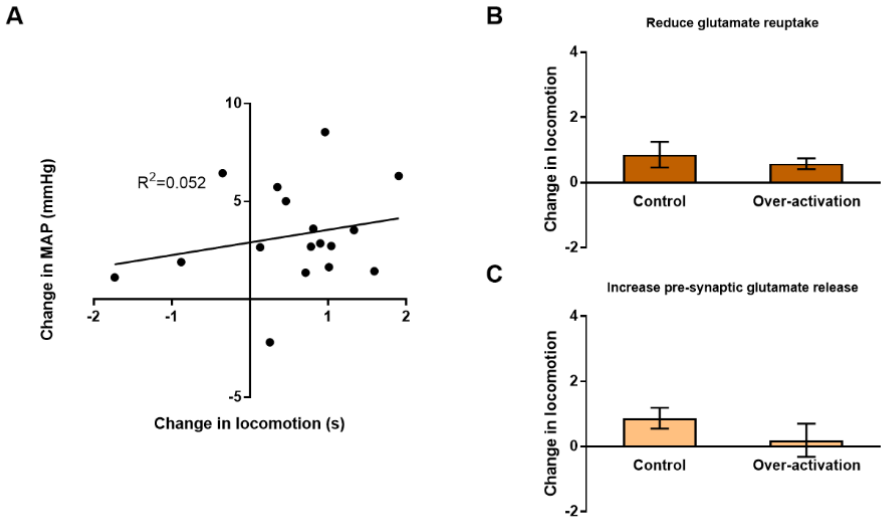


Figure 4-4 Locomotor activity during saline and drug sessions. Relevant graphs show mean ± SEM. **A** No correlation is evident between CS directed change in MAP and CS directed change in locomotion across infusion type (control, DHK and CGP/LY; R^2=0.052). **B** There was no difference in locomotion during control or over-activation by reducing glutamate reuptake (DHK; two-tailed paired *t*-test, p=0.434). **C** There was no difference in locomotion during control or over-activation by increasing pre-synaptic glutamate release (CGP/LY; two-tailed paired *t*-test, p=0.279).

Infusion	Latency (s, mean±SEM)	P value	Infusion	Latency (s, mean±SEM)	P value
sgACC/25 control	14.44 ± 2.35		pgACC/32 control	15.05 ± 4.60	
sgACC/25 DHK	23.36 ± 8.91	0.252	pgACC/32 DHK	6.03 ± 1.49	0.244
sgACC/25 CGP/LY	12.91 ± 2.95	0.485	pgACC/32 CGP/LY	13.75 ± 5.50	0.800
sgACC/25 MB	20.13 ± 7.28	0.609	pgACC/32 MB	20.13 ± 7.28	0.776

Table 4-2 Consummatory (US+) latencies to start eating food reward. P values reported from two-tailed paired *t*-tests for drug sessions vs. control (saline) sessions. MB: muscimol/ baclofen.

Both methods of over-activation caused elevations in heartrate during the 20s pre-CS baseline period, but whilst CGP/LY caused significant elevations in baseline MAP, DHK infusions did not (matching the effects reported in **Chapter 3**; **FIGURE 4-5A-D**).

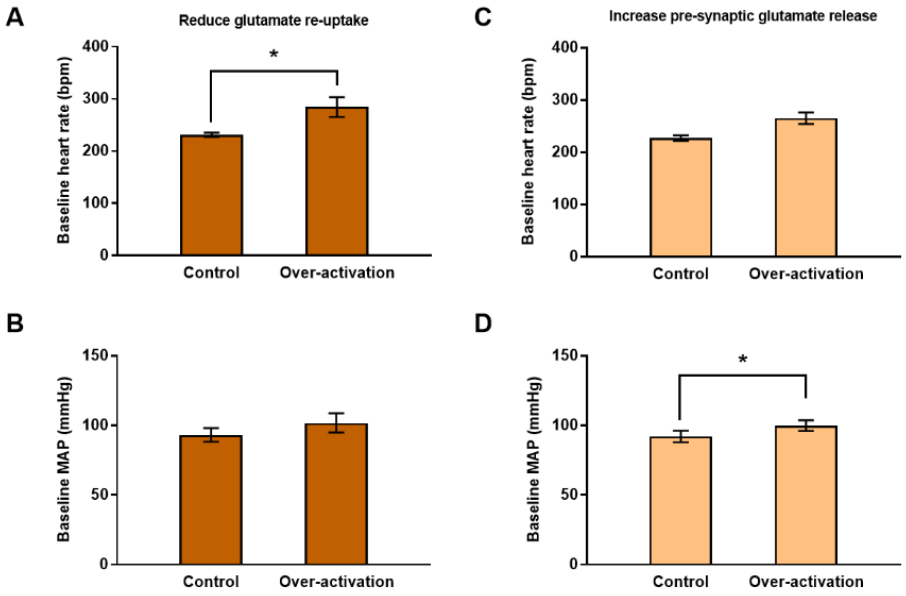


Figure 4-5 Baseline (20s period before CS) effects of sgACC/25 over-activation on HR and MAP. Relevant graphs show mean ± SEM. N=5 for reduced glutamate reuptake and inactivation. N=6 for increased pre-synaptic glutamate release. **A** Over-activation of sgACC/25 by reducing glutamate reuptake (DHK) increased baseline HR (two-tailed paired *t*-test, p=0.029). **B** Reducing glutamate reuptake had no significant effect on baseline MAP (two-tailed paired *t*-test, p=0.097). **C** Over-activation of sgACC/25 by increasing pre-synaptic glutamate release (CGP/LY) tended to increase baseline HR (two-tailed paired *t*-test, p=0.051). **D** Increasing pre-synaptic glutamate release increased baseline MAP (two-tailed paired *t*-test, p=0.014).

In contrast to sgACC/25 over-activation, we found no effect of sgACC/25 inactivation (muscimol/baclofen; $GABA_A$/$GABA_B$ receptor agonist) on arousal during either reward anticipation or consumption (**FIGURE 4-6A-D**).

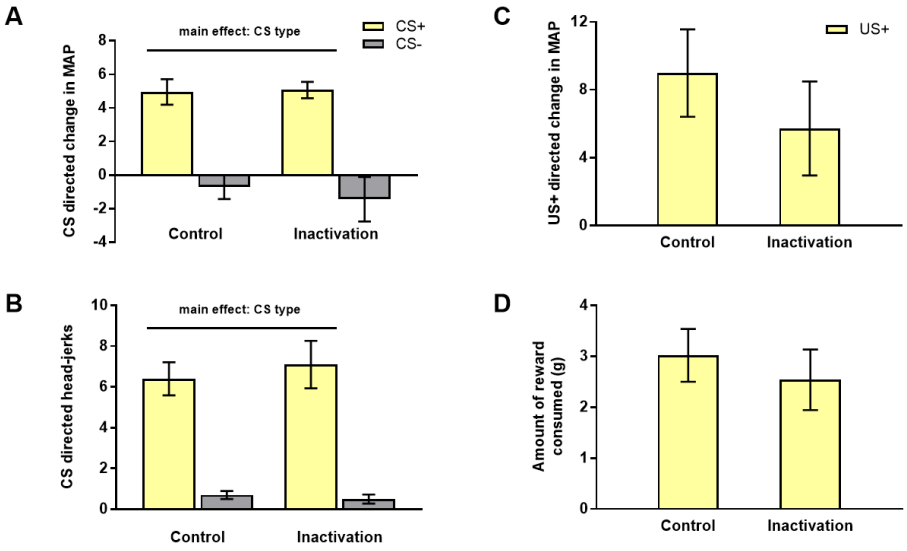


Figure 4-6 SgACC/25 inactivation had no effect on appetitive anticipatory or consummatory arousal. Relevant graphs show mean ± SEM. N=5. **A** SgACC/25 inactivation by $GABA_A$/$GABA_B$R agonism (muscimol/baclofen infusion) had no effect on anticipatory cardiovascular arousal (infusion × CS, F<1, NS; main effect of CS, $F_{1,4}$=31.76, p=0.005). **B** The same manipulation had no effect on anticipatory behavioural arousal (infusion × CS, $F_{1,4}$=1.59, p=0.276; main effect of CS, $F_{1,4}$=35.27, p=0.004). **C** There was no significant effect on consummatory cardiovascular arousal during the US+ (two-tailed paired *t*-test, p=0.226). **D** There was no significant effect on reward consumption during the US+ (two-tailed paired *t*-test, p=0.220).

Despite numerous neuroimaging studies implicating both under- and over-activity in pgACC/32 in depression and anhedonia, we found that neither bilateral pgACC/32 over-activation (using DHK or CGP/LY) nor bilateral pgACC/32 inactivation (using muscimol/baclofen) had any effect on anticipatory CS or consummatory US arousal (**FIGURE 4-7A-L**). This suggests that activity changes in pgACC/32 are not causally related to anhedonic deficits and may reflect deficits in using reward information in decision making (Amemori et al., 2015) or alternatively, compensatory changes.

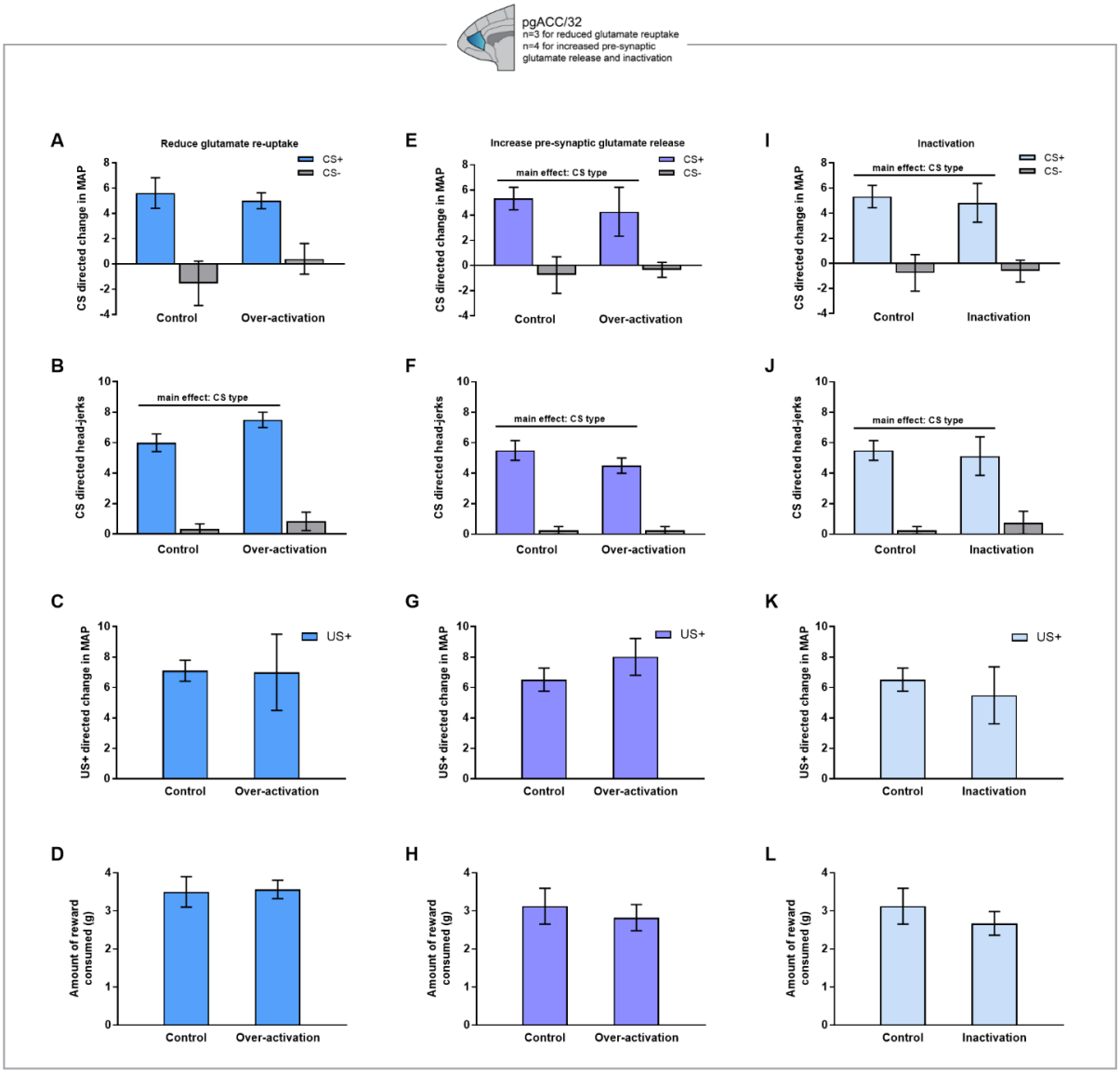


Figure 4-7 Neither pgACC/32 over-activation nor pgACC/32 inactivation impairs anticipatory or consummatory arousal. Relevant graphs show mean ± SEM. N=4 for inactivation and increased pre-synaptic release. N=3 for reduced glutamate re-uptake. **A** PgACC/32 over-activation by reducing glutamate reuptake (DHK infusion) had no effect on anticipatory cardiovascular arousal (infusion × CS, $F_{1,2}$=7.77, p=0.108; main effect of CS, $F_{1,2}$=10.07, p=0.087). **B** Reducing glutamate reuptake had no effect on anticipatory behavioural arousal (infusion × CS, F<1, NS; main effect of CS, $F_{1,2}$=342.3, p=0.003). **C** Reducing glutamate reuptake had no effect on consummatory cardiovascular arousal during the US+ (two-tailed paired *t*-test, p=0.966). **D** Reducing glutamate reuptake had no effect on reward consumption during the US+ (two-tailed paired *t*-test, p=0.742). **E** PgACC/32 over-activation by increasing pre-synaptic glutamate release (CGP52432/LY341495 infusion) had no effect on anticipatory cardiovascular arousal (infusion × CS, $F_{1,3}$=1.55, p=0.301; main effect of CS, $F_{1,3}$=11.45, p=0.043). **F** Increasing pre-synaptic glutamate release had no effect on anticipatory behavioural arousal (infusion × CS, $F_{1,3}$=6.00, p=0.092; main effect of CS,

$F_{1,3}$=63.71, p=0.004). **G** Increasing pre-synaptic glutamate release had no effect on consummatory cardiovascular arousal during the US+ (two-tailed paired *t*-test, p=0.450). **H** Increasing pre-synaptic glutamate release had no effect on reward consumption during the US+ (two-tailed paired *t*-test, p=0.484). **I** PgACC/32 inactivation by $GABA_A$/$GABA_B$R agonism (muscimol/baclofen infusion) had no effect on anticipatory cardiovascular arousal (infusion × CS, *F*<1, NS; main effect of CS, $F_{1,3}$=16.50, p=0.027). **J** Inactivation had no effect on anticipatory behavioural arousal (infusion × CS, $F_{1,3}$=1.77, p=0.275; main effect of CS, $F_{1,3}$=62.85, p=0.004). **K** Inactivation had no effect on consummatory cardiovascular arousal during the US+ (two-tailed paired *t*-test, p=0.646). **L** Inactivation had no significant effect on reward consumption during the US+ (two-tailed paired *t*-test, p=0.122).

4.4.3 SgACC/25 over-activation impairs reward motivation on a progressive ratio schedule of reinforcement

To characterize the anhedonic deficit further, the effects of over-activation of sgACC/25 (using DHK) on instrumental progressive ratio performance were assessed. Marmosets (n=3) were trained to respond to a visual stimulus on a touchscreen under increasingly demanding reinforcement requirements until a breakpoint (two minutes of inactivity) was reached (**FIGURE 4-8A, B**). Bilateral over-activation of sgACC/25 significantly impaired progressive ratio performance, reducing the breakpoint to levels significantly below both the previous day (-70.3 ± 12.5%; mean ± SEM) and control infusions (-69.5 ± 11.6%; mean ± SEM) (**FIGURE 4-8C**). This impairment was independent of individual marmosets' baseline level of responding – higher and lower responders showed similar, marked deficits (**FIGURE 4-8D**).

4.4.4 SgACC/25 over-activation has no effect on sucrose preference or consumption, despite these being common preclinical analogues of anhedonia

We also sought to investigate reward consumption in a manner directly comparable to rodent studies using a sucrose preference test adapted for marmosets (n=4; **FIGURE 4-8E**). Measurements of sucrose and water consumption were taken every 30 minutes across a two-hour testing session, with an *a priori* interest in the first 30-minute window owing to the rapid action of the intracranial infusions. In the session prior to manipulations, marmosets showed a high preference for sucrose solution over water and consumed large amounts of sucrose in both the first 30 minutes and across the two-hour testing window (**FIGURE 4-8F, G**). As a positive control, we assessed the effects of peripheral injections of the opioid antagonist naloxone – a putative modulator of the hedonic 'liking' system. In the first 30 minutes of the session, naloxone had no effect on sucrose preference (**FIGURE 4-8H**) but did reduce both sucrose consumption and water consumption (**FIGURE 4-8I**). Across the entire two-hour period, naloxone reduced sucrose consumption without affecting water consumption with the strongest effects at later timepoints (**FIGURE 4-8J**). When assessing

potential consummatory effects induced by sgACC/25 over-activation, we therefore measured both sucrose preference (reduced in rodent models of depression) and absolute sucrose consumption (reduced by naloxone and in rodent models of depression) to fully ascertain the presence of any potential consummatory impairment. Over-activation of sgACC/25 had no effect on sucrose preference or consumption in the first 30 minutes (**FIGURE 4-8K, L**) nor did it have any effect on these measures across the two-hour session (**FIGURE 4-8M**), demonstrating that whilst over-activity in this region can cause anticipatory and motivational impairments, it has no obvious effect on reward consumption.

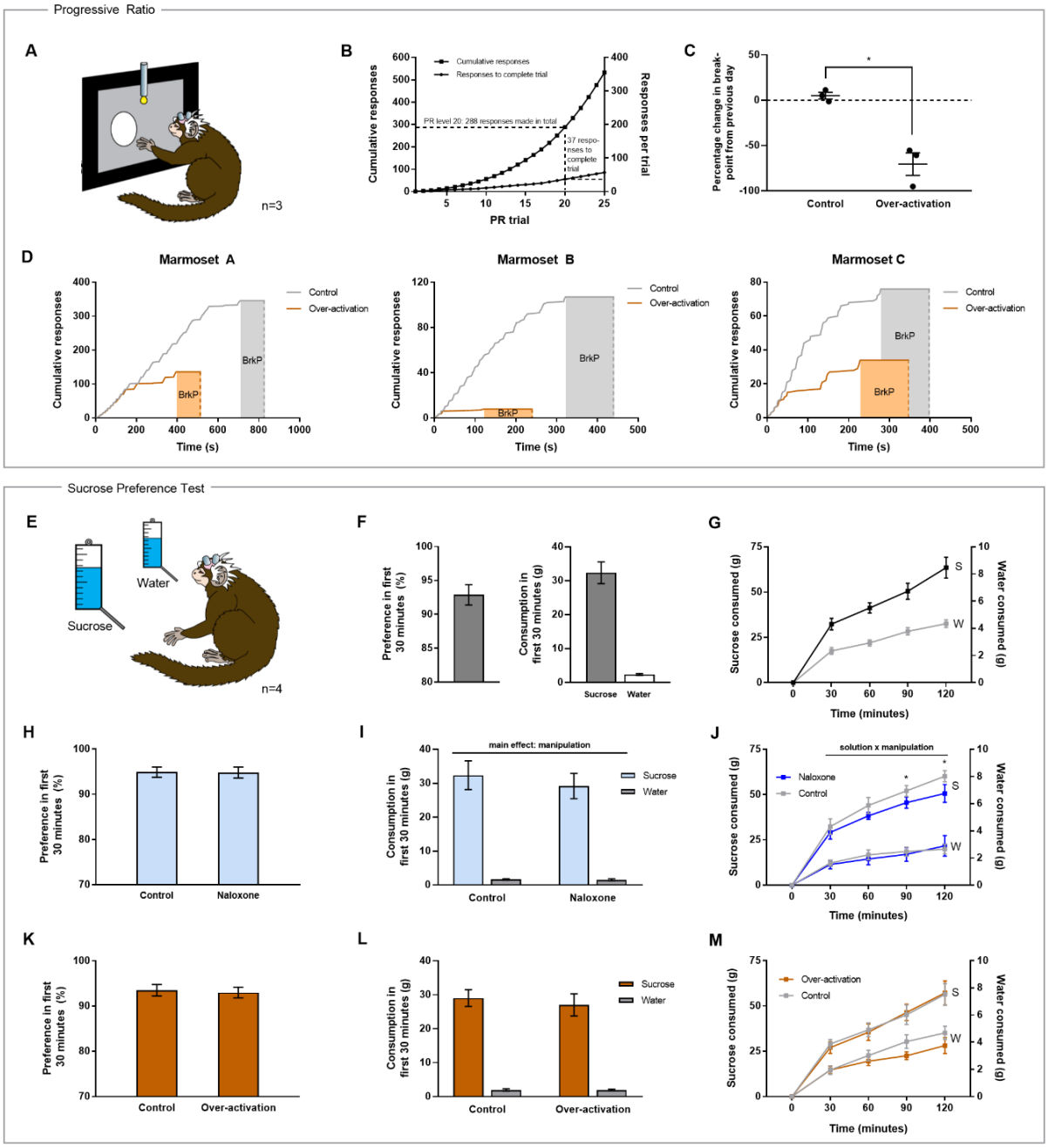


Figure 4-8 SgACC/25 over-activation impairs reward motivation on a progressive-ratio schedule of reinforcement but has no effect on sucrose preference or consumption. Relevant graphs show mean ± SEM. N=3 for progressive ratio. N=4 for sucrose preference. **A** Marmosets were trained to press a circular stimulus on a touchscreen to earn milkshake reward under increasing response demands until breakpoint was reached (two minutes with no response). **B** Task design. The response increase from trial *n* to *n*+1 starts at +1 and then doubles every eight trials until a maximum increment of +8 (+1 → +2 → +4 → +8 until end). Responses at trial 20 are highlighted. **C** SgACC/25 over-activation by reducing glutamate reuptake (DHK) decreased the number of responses marmosets made before breakpoint was reached. **D** Response profiles in control and over-activation sessions for each animal. The two-minute timeout period signifying the

breakpoint (BrkP) is shaded. **E** In the sucrose preference test, marmosets were presented with two identical bottles in their home-cage: one containing sucrose, and one containing water. A session lasted two hours with measurements taken every 30 minutes. The first 30-minute timepoint was of *a priori* interest owing to the rapid actions of the intracranial infusions. **F** Prior to experimental manipulations, marmosets showed a high preference for sucrose during the first 30 minutes of the session (92.9 ± 1.5%), consuming 32.3 ± 3.2g sucrose and 2.3 ± 0.2g water (mean ± SEM). **G** Cumulative consumption profile in the session prior to experimental manipulations. Marmosets consumed significantly more sucrose at every timepoint measured (solution [water, sucrose] × timepoint [four, 30-minute time-bins], $F_{3,9}$=26.97, p<0.0001; effect of solution, p<0.0001 at every timepoint). **H** The opioid antagonist naloxone had no effect on sucrose preference in the first 30 minutes of the session (two-tailed paired *t*-test, p=0.952). **I** Naloxone reduced both water and sucrose consumption in the first 30 minutes of the session (solution × manipulation, $F_{1,3}$=4.25, p=0.131; main effect of manipulation, $F_{1,3}$=18.00, p=0.024). **J** Across the two-hour session, naloxone reduced cumulative sucrose consumption but not cumulative water consumption (solution × manipulation, $F_{0.532,1.597}$=30.47, p=0.046). Planned comparisons conducted on sucrose and water measurements at each timepoint using Fisher's LSD test revealed a significant decrease in sucrose consumption following naloxone treatment at 90 minutes (p=0.010) and 120 minutes (p=0.024), with no significant effect on water consumption at any timepoint. **K** Over-activation of sgACC/25 by reducing glutamate reuptake had no effect on sucrose preference in the first 30 minutes of the session (two-tailed paired *t*-test, p=0.800). **L** Over-activation of sgACC/25 had no effect on sucrose or water consumption in the first 30 minutes of the session (solution × manipulation, $F_{1,3}$=1.05, p=0.381; main effect of manipulation, $F_{1,3}$=1.70, p=0.283). **M** Across the two-hour session, over-activation of sgACC/25 had no effect on cumulative sucrose or water consumption (solution × manipulation, *F*<1, NS).

4.4.5 SgACC/25 over-activation is associated with metabolic changes in a circuit including dorsomedial prefrontal cortex, dorsal anterior cingulate cortex and insula

To determine the brain regions involved in the anticipatory anhedonia induced by over-activation of sgACC/25, marmosets (n=4) underwent ^{18}F-FDG PET imaging to assess regional metabolic activity. Each subject had two counter-balanced scans: one following a saline control infusion, and one following over-activation of sgACC/25 (using DHK) (**FIGURE 4-9A**). In all cases, animals were injected with ^{18}F-FDG and then received a Pavlovian conditioning session in the test apparatus for 30 minutes before being scanned under anaesthesia (**FIGURE 4-9B**). In the voxel-based analysis, a subtraction image was produced (i) for (over-activation – control) to determine brain regions which were over-active following sgACC/25 over-activation; and (ii) for (control – over-activation) to determine brain regions which were under-active. In parallel, we obtained cardiovascular (n=3 owing to one telemetry probe failure) and behavioural (n=4) readouts during the PET conditioning session

immediately prior to the scan to confirm whether the manipulations replicated the anticipatory anhedonia described in **4.4.2**.

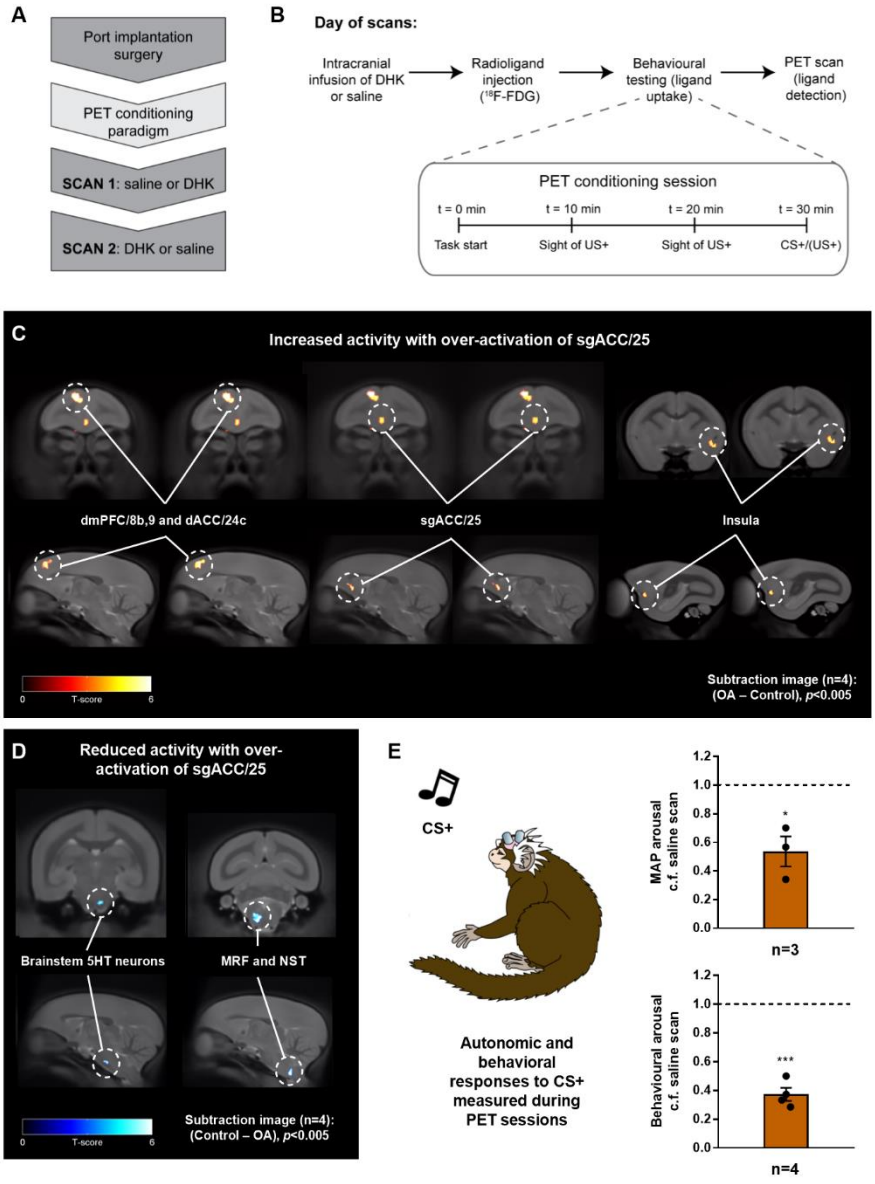


Figure 4-9 ^{18}F-FDG PET imaging revealed metabolic changes in a network of brain regions associated with interoception and reward processing following sgACC/25 over-activation. Relevant graphs show mean ± SEM. N=3 for cardiovascular arousal. N=4 for behavioural arousal. N=4 for all PET images; clusters discussed are significant at the level of p<0.005 with an extent threshold adjusted for search volume of 26 voxels. **A** Following implantation of a subcutaneous port into the internal jugular vein, marmosets were trained on a modified version of the appetitive Pavlovian conditioning paradigm (see **B**) in preparation for scanning. Saline control and DHK scans were counterbalanced. **B** On the day of a scan, animals received an infusion of DHK or saline immediately followed by radioligand injection through the port. The PET conditioning session (inset) lasted 30 minutes (to facilitate adequate ligand uptake), consisting of two 20s periods of the sight of

reward without access, and a final 20s CS+ period. During training, the CS+ was followed by a 120s US+. On scan days, the animals were immediately removed from the apparatus when the CS+ period terminated, anaesthetised and then scanned. **C** Subtraction images calculated from voxelwise subtraction of SUVRc values for over-activation (OA) scans – saline control scans, showing brain regions with increased activity following sgACC/25 over-activation. Increased metabolic activity was observed in sgACC/25 (centre), together with a region of dmPFC spanning dmPFC/8b,9 and dACC/24c (surviving $p<0.001$). More caudally, increased metabolic activity was observed in the left ventral insula. **D** Subtraction images calculated from voxelwise subtraction of SUVRc values for saline control scans – OA scans, showing brain regions with reduced activity following sgACC/25 over-activation. Reduced metabolic activity was observed in a region encompassing brainstem 5HT neurons and, more caudally, brainstem autonomic control centres including the NTS and MRF. **E** Cardiovascular and behavioural responses were measured during the CS+ period in the PET conditioning sessions immediately prior to scanning. Compared to saline scans, over-activation of sgACC/25 significantly blunted cardiovascular (ratio of MAP response to saline scans; one-sample *t*-test to 1.0, $p=0.048$) and behavioural (ratio of head-jerk response to saline scans; one sample *t*-test to 1.0, $p<0.001$) arousal.

PET imaging revealed that over-activation of sgACC/25 increased ^{18}F-FDG uptake in sgACC/25 (confirming that the drug manipulation increases metabolism in sgACC/25) together with significant increases in uptake in dmPFC/8b,9, dACC/24c and left ventral insula (**FIGURE 4-9C**). SgACC/25 over-activation also lowered metabolic activity in a brainstem region encompassing components of the serotonergic raphe nuclei (rostral group B9), the NTS and the medullary reticular formation (MRF, **FIGURE 4-9D**). Importantly, over-activation of sgACC/25 on the day of scanning replicated the reduction in behavioural and cardiovascular appetitive arousal during CS+ presentation (**FIGURE 4-9E**).

4.4.6 Acute administration of ketamine, but not citalopram, reverses anticipatory anhedonia induced by over-activation of sgACC/25

To determine whether the novel antidepressant ketamine could reverse symptoms of anhedonia induced by over-activation of sgACC/25, marmosets (n=4) received a single intramuscular injection of ketamine (0.5mg/kg) followed by over-activation of sgACC/25 (using DHK) at 4 hours, 1 day and 7 days after injection whilst undergoing behavioural testing on the appetitive Pavlovian conditioning paradigm (**FIGURE 4-10A**). These time-points were chosen to coincide with clinical literature showing rapid (4 hour time-point) and relatively sustained (1 day and 7 day time-points) effects of a single acute administration of ketamine to improve scores on depression scales (Abdallah et al., 2015). In three animals, we also determined the endpoint of ketamine's action.

In a control experiment, ketamine alone (in the absence of sgACC/25 over-activation) had no effect on either autonomic or behavioural components of appetitive arousal compared to

vehicle control (**FIGURE 4-10B**). Whilst over-activation of sgACC/25 4 hours following ketamine injection still resulted in anticipatory anhedonia, over-activation at 1 day and 7 days post-injection did not: despite receiving infusions of DHK, animals showed a MAP rise and head-jerking response selectively to the CS+ (**FIGURE 4-10C**). Therefore, at these time-points, ketamine successfully reversed the anticipatory anhedonic deficit induced by over-activation of sgACC/25. In two of the three animals where the end-point was assessed, ketamine's action had abrogated by three weeks; in the third animal, by four weeks (indicated by the return of the over-activation induced blunting of CS+ arousal).

We also determined the sensitivity of the anticipatory anhedonia to an acute dose of the first-line SSRI antidepressant citalopram (10mg/kg; **FIGURE 4-10D**). Acute citalopram has been shown to have rapid and profound effects on marmosets' responsivity to a human intruder (Santangelo et al., 2016). In a control experiment, an intramuscular injection of citalopram in the absence of sgACC/25 over-activation had no effect on either autonomic or behavioural components of appetitive arousal (**FIGURE 4-10E**). Unlike ketamine, acute citalopram administration failed to reverse either the autonomic or behavioural components of over-activation induced anticipatory deficit, suggesting that acute doses are ineffective in treating the anticipatory anhedonia (**FIGURE 4-10F**).

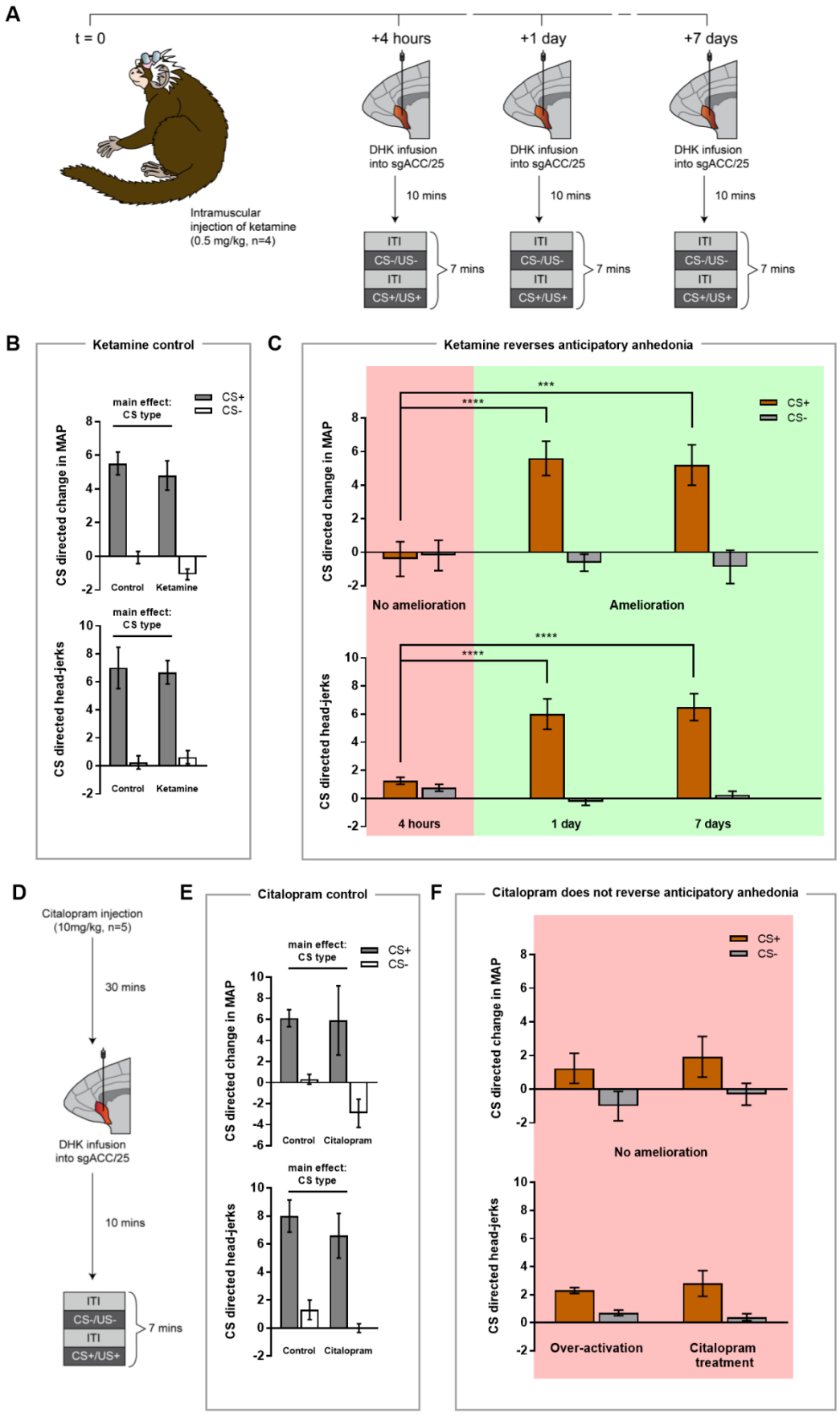

A
t = 0
+4 hours
+1 day
+7 days
Intramuscular injection of ketamine (0.5 mg/kg, n=4)
DHK infusion into sgACC/25
10 mins
ITI
CS-/US-
ITI
CS+/US+
7 mins
B
Ketamine control
main effect: CS type
CS+
CS-
CS directed change in MAP
CS directed head-jerks
Control
Ketamine
C
Ketamine reverses anticipatory anhedonia
***
****
No amelioration
Amelioration
4 hours
1 day
7 days
D
Citalopram injection (10mg/kg, n=5)
30 mins
E
Citalopram control
Control
Citalopram
F
Citalopram does not reverse anticipatory anhedonia
Over-activation
Citalopram treatment

Figure 4-10 A single intramuscular injection of ketamine ameliorates the cardiovascular and behavioural anticipatory anhedonia induced by over-activation sgACC/25 in a time-dependent manner – whereas acute citalopram has no effect. Relevant graphs show mean ± SEM. N=4 for ketamine study. N=5 for citalopram study. **A** Timeline of ketamine study. Marmosets received a single intramuscular injection of ketamine (t=0) followed by over-activation of sgACC/25 (DHK) 4 hours, 1 day and 7 days later. **B** Ketamine alone (CS-/CS+ sessions in between DHK timepoints) had no effect on cardiovascular (infusion × CS, F<1, NS; main effect of CS maintained, $F_{1,3}$=86.5, p=0.003) or behavioural (infusion × CS, $F_{1,3}$<1; main effect of CS maintained, $F_{1,3}$=31.69, p=0.011) responses. **C** Ketamine had a time-dependent effect to reverse the cardiovascular (timepoint × CS, $F_{2,12}$=14.71, p<0.001) and behavioural (timepoint × CS, $F_{2,12}$=19.59, p<0.001) aspects of the anticipatory anhedonia induced by sgACC/25 over-activation (DHK infusion). Compared to control infusions of saline vehicle (not shown), sgACC/25 over-activation 4 hours after ketamine administration still resulted in significant blunting of cardiovascular (infusion × CS, $F_{1,3}$=60.46, p=0.004; effect of infusion on CS+, p=0.003) and behavioural (infusion × CS, $F_{1,3}$=25.59, p=0.015; effect of infusion on CS+, p=0.012) arousal. Over-activation 1 day following ketamine administration evidenced amelioration of the cardiovascular (4 hours *vs.* 1 day: CS+, p<0.0001; CS-, p=0.863) and behavioural (4 hours *vs.* 1 day: CS+, p<0.0001; CS-, p=0.371) impairments compared to 4 hours. Similarly, over-activation 7 days following ketamine administration evidenced amelioration of the cardiovascular (4 hours *vs.* 7 days: CS+, p<0.001; CS-, p=0.704) and behavioural (4 hours *vs.* 7 days: CS+, p<0.0001; CS-, p=0.767) impairments compared to 4 hours. **D** Timeline of acute citalopram study. Marmosets received a single intramuscular injection of citalopram followed by over-activation of sgACC/25 (DHK) 30 minutes later. **E** Citalopram alone had no effect on cardiovascular (infusion × CS, $F_{1,4}$=1.17, p=0.340; main effect of CS, $F_{1,4}$=19.39, p=0.012) or behavioural (infusion × CS, F<1, NS; main effect of CS, $F_{1,4}$=30.29, p=0.005) arousal. **F** Compared to sgACC/25 over-activation alone, acute citalopram had no effect on the cardiovascular (infusion × CS, F<1, NS) or behavioural (infusion × CS, $F_{1,4}$=1.19, p=0.338) components of anticipatory anhedonia. Compared to control infusions of saline vehicle (not shown), sgACC/25 over-activation with acute citalopram still resulted in significant blunting of cardiovascular (infusion × CS, $F_{1,4}$=8.74, p=0.042; effect of infusion on CS+, p=0.016) and behavioural (infusion × CS, $F_{1,4}$=462, p<0.0001; effect of infusion on CS+, p<0.0001) arousal.

4.4.7 Reversal of anticipatory anhedonia by ketamine is associated with normalization of metabolic activity in dmPFC and dACC, and deactivation of the insula

Marmosets (n=4) received an additional third scan consisting of sgACC/25 over-activation (using DHK) following an injection of ketamine 1 day earlier, coinciding with a timepoint at which anticipatory anhedonia was reversed. Subtraction images were computed for (i) (over-activation – [over-activation + ketamine]) to determine brain regions showing decreased metabolic activity following administration of ketamine; and (ii) ([over-activation + ketamine] –

over-activation) to determine brain regions showing increased activity following administration of ketamine. Ketamine administration 1 day prior to scanning reversed metabolic changes within the dmPFC, dACC and left ventral insula (corresponding to the regions showing elevated activity following sgACC/25 over-activation; **FIGURE 4-11A**). No prefrontal or subcortical regions showed significant increases in activity.

To determine whether ketamine normalized metabolic activity to control levels or diminished activity below control levels, a (iii) third subtraction image was calculated for (control – [over-activation + ketamine]). Results from this comparison show that whilst activity in dmPFC/dACC returned to control levels, activity in the left insula was reduced below activity levels observed in the control condition (**FIGURE 4-11B**). Therefore, in the context of sgACC/25 over-activation, ketamine normalises activity within dmPFC/dACC but deactivates the left insula. Cardiovascular and behavioural data obtained on the day of scanning showed that ketamine successfully reversed the DHK-induced anticipatory anhedonic deficit (**FIGURE 4-11C**).

Using the stringent criterion applied in the voxel-based approach, there was no apparent effect of ketamine on metabolic activity in sgACC/25 itself. However, using an atlas-defined region of interest (ROI) (Paxinos et al., 2011), we examined the mean ^{18}F-FDG uptake in sgACC/25 across control, over-activation and [over-activation + ketamine] conditions to determine if the beneficial effect of ketamine depends – at least in part – on modulation of sgACC/25 activity in response to DHK-induced reductions in glutamate reuptake. Across all four subjects, we found that ketamine administration reduced the increased metabolic activity associated with DHK infusions into sgACC/25 (). These data suggest that the efficacy of ketamine is related to (likely neuroplastic-mediated) alterations in the responsivity of sgACC/25 to elevated levels of extracellular glutamate.

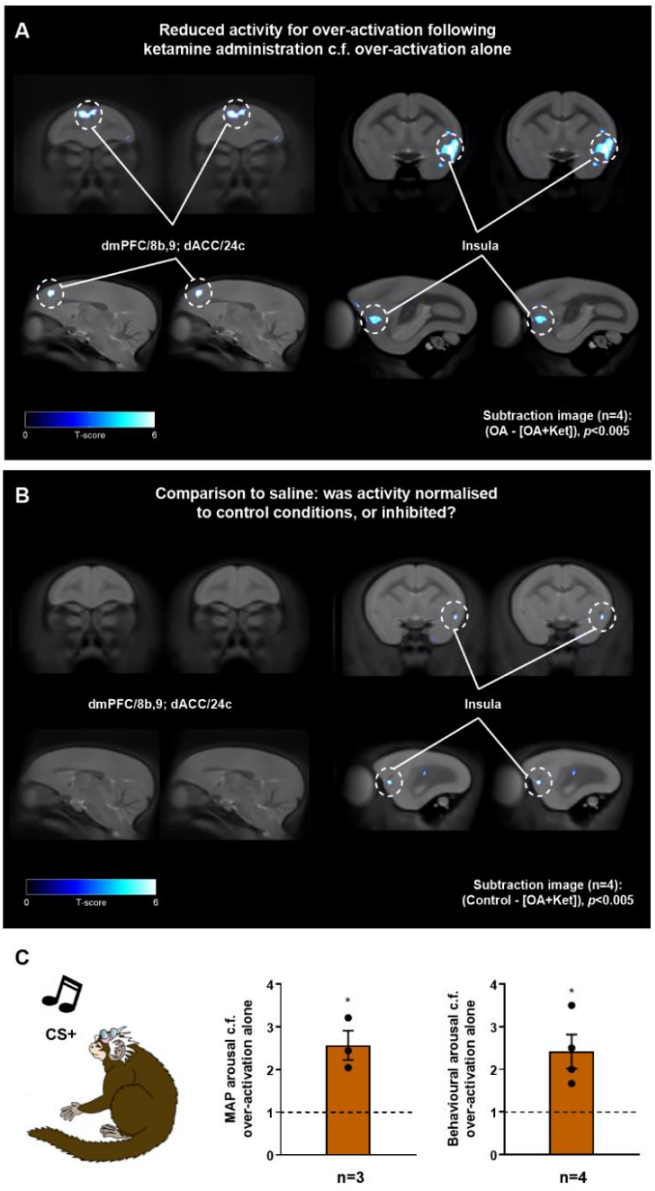


Figure 4-11 Reversal of anticipatory anhedonia by ketamine is associated with metabolic changes within dmPFC, dACC and insula. Relevant graphs show mean ± SEM. N=3 for cardiovascular arousal. N=4 for behavioural arousal. N=4 for all PET images; clusters are significant at the level of p<0.005 with an extent threshold of 26 voxels. **A** Subtraction images calculated for over-activation (OA) – [OA + ketamine (Ket)] scans, showing regions with reduced activation following ketamine 1 day earlier. Reversal of anticipatory anhedonia was associated with reduced activity in dmPFC, dACC and left insula. **B** Subtraction images calculated for control– [OA + ketamine] scans revealed that activity in the dmPFC/8b,9 and dACC/24c region was no different from control scans, indicating that ketamine had normalised over-activity in these regions to control levels. However, activity in the left insula was reduced even compared to control conditions, suggesting that ketamine administration caused deactivation of the insula. **C** On the day of scanning, ketamine administration 1 day earlier ameliorated blunted cardiovascular (ratio of MAP response, one-sample *t*-test to 1.0, p=0.017) and behavioural (ratio of head-jerk response, one sample *t*-test compared to 1.0, p=0.038) arousal compared to over-activation alone.

Manipulation	Hemisphere	sgACC/25 SUVR values by subject				Mean ± SEM
		□	▽	◇	◑	
Control	Left	0.856	1.028	0.843	0.822	0.887 ± 0.047
	Right	0.785	1.002	0.842	0.833	0.866 ± 0.047
Over-activation	Left	1.069	1.157	0.921	1.019	1.041 ± 0.049
	Right	1.023	1.040	0.933	0.992	0.997 ± 0.023
Over-activation + Ketamine	Left	1.017	0.974	0.882	0.807	0.920 ± 0.047
	Right	0.967	0.905	0.880	0.807	0.890 ± 0.033

Table 4-3 Measurements of SUVR changes across control, over-activation and [over-activation + ketamine] in an atlas-defined sgACC/25 ROI. Within this ROI, there was a significant effect of manipulation on SUVR values (manipulation × hemisphere, $F_{2,6}$<1, NS; effect of manipulation: $F_{2,6}$=6.22, p=0.034). Planned comparisons using Fisher's LSD test revealed a significant difference between control *vs.* over-activation (p=0.016) and over-activation *vs.* [over-activation + ketamine] (p=0.037) conditions, but not for control *vs.* [over-activation + ketamine] (p=0.530) conditions.

4.5 Discussion

Despite being a core symptom of depression, anhedonia is poorly characterised, and its neurobiological basis remains unknown, severely retarding the development of effective treatments. In the present study, we addressed these issues using interventional manipulations in marmosets to causally implicate sub-regions of the vmPFC in precisely defined subtypes of anhedonia (anticipatory *vs.* motivational *vs.* consummatory). We show that over-activity in marmoset sgACC/25 (but not over-activity or reduced activity in pgACC/32) selectively blunts anticipatory arousal for reward without affecting its consumption, and profoundly diminishes motivation for reward.

4.5.1 Fractionating anhedonia

Over-activation of marmoset sgACC/25 – achieved with two different methods (reducing glutamate reuptake and increasing pre-synaptic glutamate release) – blunted anticipatory cardiovascular and behavioural responses to a cue predicting food reward (CS+) but did not robustly affect cardiovascular or behavioural responses associated with consumption of the reward (US+). As both methods reduce anticipatory arousal, this suggests that elevated glutamate levels within sgACC/25 are sufficient to cause anticipatory anhedonia independent of the precise mechanism through which these increases occur. However, whether the effects of elevated glutamate levels are through increasing activity in pyramidal output neurons or inhibitory interneurons within sgACC/25 remains unclear. Furthermore, through actions at presynaptic mGluRs, elevated glutamate levels may locally suppress certain clusters of neurons. Further immunohistochemical work coupled with cFos expression may clarify the population of neurons and the pattern in which these cells are affected by the pharmacological manipulations described in this chapter.

SgACC/25 over-activation also diminished appetitive motivation as assessed by reduced breakpoints on a progressive ratio task. Whether it is possible to separate anticipatory and motivational impairments remains unclear. Impairments in Pavlovian reward anticipation impact upon instrumental performance through Pavlovian-to-instrumental transfer, conditioned reinforcement and conditioned approach (Dickinson and Balleine, 1994; Holland, 1977; Mackintosh, 1974). In depressed patients, deficits in reward motivation are thought to be driven by a primary decrease in anticipatory pleasure (Sherdell et al., 2012). Therefore, motivational impairments may not result from deficits in goal-directed performance per se; rather, from a reduced influence of Pavlovian cues signalling reward which would otherwise support responding.

Whilst we found no impact of sgACC/25 over-activation on behavioural or cardiovascular consummatory arousal, the measurement of reward consumption in the Pavlovian conditioning paradigm (arousal during two minutes of consumption of high incentive food) is

different to consumption assessed in the rodent sucrose preference test which is typically measured over a longer time period (Ferenczi et al., 2016; Tye et al., 2013). Thus, we also determined if any effect of sgACC/25 over-activation could be detected on a version of the sucrose preference test adapted for marmosets. We measured both sucrose consumption (which was significantly reduced by the opioid antagonist naloxone acting as a positive control) and sucrose preference (both measures being affected by rodent models of depression) to fully characterize marmosets' consummatory profile following sgACC/25 manipulations. SgACC/25 over-activation had no effect on either sucrose consumption or sucrose preference, providing no support for an involvement of sgACC/25 in consummatory anhedonia.

These data illustrate that the transient anhedonia induced by pharmacological over-activation of sgACC/25 possesses face validity when compared to the anhedonic state observed in depressed patients, who typically show anticipatory and motivational deficits rather than consummatory ones (Forbes et al., 2009; Klein, 1987; McFarland and Klein, 2009; Smoski et al., 2009). The contrasting findings across different reward domains illustrates the importance of careful consideration of the psychological constructs impaired in psychiatric disorders. Furthermore, these findings suggest that the sucrose preference test is insufficient if used in isolation – sgACC/25 over-activity blunts aspects of reward processing not measured by this test.

The anhedonic deficits displayed were not due to a general blunting of emotional arousal since the same manipulation induces heightened behavioural arousal to an HI (see **Chapter 3**). This highlights the opposing effects of sgACC/25 over-activity across emotional domains and implicates this region in adapting behaviour to emotional context. In addition, these data implicate sgACC/25 over-activity in symptoms of anxiety which commonly manifest in depressed patients (Kessler et al., 2003). Indeed, several studies have identified elevated activity in a subgenual region (including area 25) associated with sustained and unpredictable threat (Alvarez et al., 2011; Hasler et al., 2007b).

4.5.2 Circuit-wide changes associated with over-activation induced anhedonia

To characterize the circuit affected by sgACC/25 over-activation, we used ^{18}F-FDG PET imaging combined with intracerebral microinfusions. PET imaging revealed increased metabolic activity in sgACC/25, dmPFC/dACC and left ventral insula following over-activation of sgACC/25. Elevated connectivity between these regions has been observed in depressed populations (Connolly et al., 2013; Sheline et al., 2010) but is seldom related to anhedonia. Nevertheless, the increase in dmPFC/dACC activity is consistent with previously reported results of increased activity in a similar region during reward anticipation in currently depressed or remitted patients compared to controls (Dichter et al., 2012; Gorka et al., 2014;

Knutson et al., 2008). Similarly, an over-active insula has been observed during the anticipation of rewards in groups at high-risk of depression (Gotlib et al., 2010) and following presentation of positively-valenced pictures in depressed patients (Mitterschiffthaler et al., 2003).

Reduced activity following sgACC/25 over-activation was seen in a region encompassing brainstem 5HT neurons (B9 group of raphe nuclei). The importance of interplay between vmPFC and 5HT neurons has been appreciated in terms of stress controllability (Amat et al., 2005), but more recently a role for 5HT neuronal signalling during reward anticipation has been demonstrated (Li et al., 2016). More caudally, we observed reduced activity in autonomic control centres in the region of the NST. Several tract tracing studies have identified connectivity between primate vmPFC, hypothalamus and autonomic effector regions in the brainstem (Ghashghaei and Barbas, 2002; Joyce and Barbas, 2018; Rempel-Clower and Barbas, 1998) through which regions including sgACC/25 can modulate autonomic function during baseline and task conditions (Rudebeck et al., 2014; Wallis et al., 2017).

4.5.3 Ketamine as an efficacious treatment for over-activation induced anhedonia

Ketamine has emerged as a fast-acting antidepressant with efficacy in otherwise treatment resistant cases (Berman et al., 2000; Murrough et al., 2013b). Unlike current first-line medication, treatment with ketamine ameliorates reward-related dysfunction in both bipolar and unipolar depression (Lally et al., 2014, 2015). Whilst clinical studies have observed variable antidepressant effects within 4 hours of treatment, consistent effects are observed 1 day later which are sustained for 3-7 days (Abdallah et al., 2015). Here, we show that ketamine did reverse anticipatory cardiovascular and behavioural anhedonia induced specifically by sgACC/25 over-activation. We did not observe an anti-anhedonic effect of ketamine at 4 hours, but ketamine did reverse the over-activation induced anticipatory deficit 1 day and 7 days following administration. This supports the hypothesis that the anti-anhedonic effects of ketamine are contingent upon neuroplastic mechanisms rather than acute changes in glutamate levels associated with antagonism of NMDA receptors (Melo et al., 2015).

Ketamine not only ameliorated the anticipatory anhedonia induced by sgACC/25 over-activation but also reversed the associated elevated activity within sgACC/25, dmPFC/dACC and left ventral insula. Activity in the former two regions was normalized, whereas activity in the insula was inhibited below control levels. Reduced activity within sgACC/25 itself is consistent with neuroimaging studies which have shown that successful treatment of depression using SSRIs is associated with reduced activity within sgACC/25 (Mayberg et al., 2000). Normalisation of activity in dmPFC/dACC following ketamine differs from a recent

clinical study in which the efficacious action of ketamine was associated with increased (rather than reduced) metabolism in these same regions (Lally et al., 2015). However, these opposing effects may be related to ketamine's actions at different timepoints: patients in Lally *et al.* were imaged 2 hours following ketamine administration, whereas in the present study marmosets were imaged 24 after administration. The rapid *vs.* slow actions of ketamine are associated with different effects on neural circuitry, increasing dACC-mPFC functional connectivity acutely (Grimm et al., 2015) but decreasing it 24 hours later (Scheidegger et al., 2012).

4.5.4 Translational considerations

This study was designed to address several challenges faced in the translation of preclinical studies to humans: specifically, issues of homology; issues of symptom heterogeneity; and issues concerning the quantification of emotion.

Firstly, concerning homology, the important contributions that NHP studies make to understanding prefrontal dysfunction in psychiatric disorders are exemplified herein. The high degree of cytoarchitectonic similarity between marmoset and human vmPFC (Burman and Rosa, 2009) means marmosets are an ideal species to parcellate the contributions of vmPFC subregions, including sgACC/25, to symptoms of depression. The putative anatomical homologue of primate sgACC/25 is rodent IL (Heilbronner et al., 2016), activations of which have been shown to reduce appetitive motivation (John et al., 2012) (although see (Gasull-Camós et al., 2017)). However, whether primate/rodent homology necessarily implies functional analogy is far from clear – indeed, we have shown opposite effects on the regulation of negative emotion following inactivations of marmoset sgACC/25 to those seen in rodent IL studies (Wallis et al., 2017). Secondly, concerning symptom heterogeneity, the impairments in anticipatory and motivational – but not consummatory – domains provides neurobiological evidence for the fractionation of anhedonia into separable subtypes. It is imperative that these subtypes are recognized both preclinically and clinically: depressed patients may present with selective impairments with distinct underlying neural mechanisms and hence differing optimal treatment strategies. Finally, anhedonia is a complex emotional construct consisting of behavioural and physiological changes that cannot be adequately measured using single experimental outputs. Whilst informative, studies examining subjective, autonomic or behavioural components of emotion in isolation fail to account for the complex nature of emotion. Future work must delineate the precise psychological and physiological functions that sgACC/25 subserves in the regulation of positive and negative emotion and isolate the pathophysiological processes that can lead to chronic elevations in sgACC/25 activity associated with mood disorders.

4.6 Conclusion

The insights into sgACC/25 dysfunction gained from this study have wide-ranging implications for both preclinical and clinical research into reward-processing deficits in depression. The significance of an over-active sgACC/25 has – until now – been completely unexplored in the context of anticipatory and motivational anhedonia, yet we have revealed a critical causal role for this region in blunted reward processing characteristic of the anhedonic state and revealed the network of altered activity induced by such over-activation. Furthermore, these results highlight the anti-anhedonic effects of ketamine through reversal of anticipatory anhedonic impairments induced specifically by over-activation of sgACC/25. As well as progressing our understanding of treatment strategies in depression, by employing a multifaceted approach to quantify emotion in marmosets we have made significant progress in bridging the translational divide between rodents and humans. Overall, this study demonstrates the critical role that interventional studies in primates must play to further our understanding of the neurobiological mechanisms underlying symptoms of emotion dysregulation characteristic of psychiatric disorders.

5 ENHANCED CARDIOVASCULAR AND BEHAVIOURAL CORRELATES OF NEGATIVE EMOTION INDUCED BY OVER-ACTIVATING PRIMATE SGACC/25

Abbreviation	Meaning
^{18}F-FDG PET	^{18}Fluorine-fluorodeoxyglucose positron emission tomography
ANOVA	Analysis of variance
BA	Brodmann area
CRH	Corticotropin releasing hormone
CS	Conditioned stimulus
dACC	Dorsal anterior cingulate cortex
DHK	Dihydrokainic acid
EAAT2	Excitatory amino acid transporter-2
EFA	Exploratory factor analysis
GAD	Generalised anxiety disorder
GCR	Glucocorticoid receptor
HARS	Hamilton Anxiety Rating Scale
HI	Human intruder
HR	Heart rate
IL	Infralimbic (cortex)
ITI	Inter-trial interval
KMO	Kaiser-Meyer-Olkin
MAP	Mean arterial pressure
mPFC	Medial prefrontal cortex
NMDA	*N*-methyl-*D*-aspartate (receptor)
NS	Not significant
pgACC	Perigenual anterior cingulate cortex
PTSD	Post-traumatic stress disorder
SAD	Social anxiety disorder
SEM	Standard error of the mean
sgACC	Subgenual anterior cingulate cortex
TSAB	Time spent at back
TSAF	Time spent at front
US	Unconditioned stimulus
vmPFC	Ventromedial prefrontal cortex
VS	Vigilant scanning

5.1 Abstract

A cardinal feature of depression is enhanced negative emotion, which has been fractionated into several inter-related constructs: low mood, despair and learned helplessness, enhanced intolerance of uncertainty and, in some cases, impaired fear regulation. These clusters overlap with patterns of dysfunction seen in anxiety disorders – perhaps unsurprising, given that these disorders are highly co-morbid (Gorman, 1996; Lamers et al., 2011; Pollack, 2005). Whilst elevated activity within sgACC/25 has been implicated in negative emotion and mood disorders – together with normalisation of this activity following successful treatment – whether these activity changes are causally related to increases in negative affect remains unknown. Here we combine targeted intracerebral microinfusions with cardiovascular and behavioural monitoring in marmoset monkeys to show that over-activation of sgACC/25 heightens behavioural and cardiovascular arousal in aversive contexts, elevates circulating cortisol levels and appears to blunt stress recovery. The same manipulation elevates anxiety responses to an uncertain threat in the form of an unfamiliar human. When tested for its ability to reverse enhanced intolerance of uncertainty following sgACC/25 over-activation, ketamine failed to reverse the impairments, suggesting differential efficacy in treating reward-related (**Chapter 4**) and anxiety-related symptoms.

5.2 Introduction

Having established the effects of sgACC/25 over-activity on appetitive behavioural and cardiovascular arousal (**Chapter 4**), the experiments described in this chapter aimed to determine the effects of sgACC/25 over-activity on negative emotion. Enhanced negative emotion is a feature of mood and anxiety disorders, which are themselves highly co-morbid (Gorman, 1996; Lamers et al., 2011; Pollack, 2005). Although difficult to parcellate, elevated negative emotion can be conceptualised as consisting of enhanced low mood (subjective sadness), despair and learned helplessness, impaired fear regulation (for example, fear generalisation, impaired fear extinction and impaired recovery following stressors) and enhanced intolerance of uncertainty. The former component is intractable with animal studies, whereas aspects of the latter three are (**Figure 5-1**). The work described in this chapter will focus on two of these aspects: fear regulation – as indexed by fear generalisation, extinction and stress-recovery – and intolerance of uncertainty.

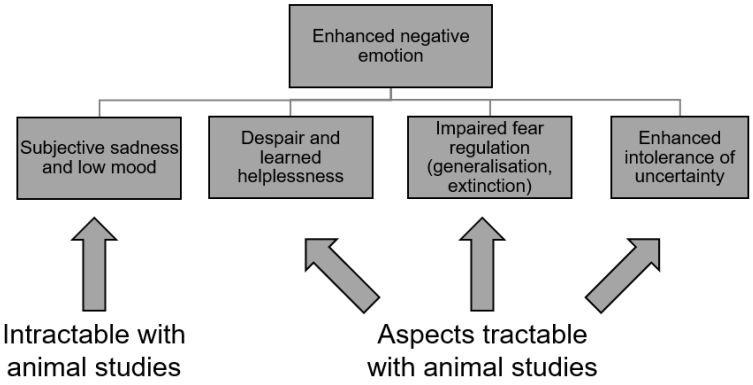


Figure 5-1 Tractability of constructs in negative emotion with animal studies. Psychiatric diseases such as mood and anxiety disorders are typified by enhanced negative emotion. Enhanced negative emotion is multi-faceted, including subjective sadness, despair and learned helplessness, intolerance of uncertainty and impairments in fear regulation. Whilst the subjective components of enhanced negative affect cannot be assessed using animal studies, aspects of learned helplessness, enhanced intolerance of uncertainty and impairments in fear regulation can be measured behaviourally.

Fear generalisation and impairments in fear extinction associated with impaired fear regulation are most strongly related to anxiety disorders. Excessive fear generalisation has been observed in patients with PTSD, GAD and panic disorder (Anastasides et al., 2015; Dymond et al., 2015; Lissek et al., 2010), and the propensity to generalise fear responses during fear conditioning procedures has been linked to high trait levels of anxiety (but not low mood) in healthy controls (Park et al., 2018). State and trait anxiety have also been linked to impaired fear extinction, with patients/high trait-anxious controls typically showing slower CS-noUS learning (Dibbets and Evers, 2017; Milad et al., 2014). Although fear extinction has rarely been studied in depressed populations, preliminary studies suggest that fear extinction is unaffected by symptoms of depression (Dibbets et al., 2015). Neurobiologically, several studies link vmPFC activity to fear regulation – specifically, in fear generalisation (Greenberg et al., 2013; Lissek et al., 2014) and in the recall of fear extinction (Milad et al., 2007a; Phelps et al., 2004).

One aspect of impaired fear regulation that may be transdiagnostic across anxiety *and* depression is blunted stress recovery. Recovery from stress has received comparatively little attention compared to responses during stress itself (Linden et al., 1997). However, a meta-analysis comparing cortisol dynamics in healthy controls *vs.* depressed patients has shown similar baseline and stress-induced levels of cortisol between groups, much higher cortisol levels in depressed patients during recovery (Burke et al., 2005) which has been linked to

rumination (LeMoult and Joormann, 2014). Similarly, trait and state anxious[3] individuals show slower physiological and subjective recovery from stress (Willmann et al., 2012). A large region of vmPFC (throughout its rostrocaudal extent, including BA10, 14 and 25) is associated with positive emotions during stress recovery, supporting a role for this region in top-down regulation of emotion generating structures following stress exposure (Yang et al., 2018a). Several studies have also implicated vmPFC activity in the related role of signalling stress controllability (Bhanji and Delgado, 2014; Sinha et al., 2016).

Intolerance of uncertainty is "*a dispositional characteristic that results from a set of negative beliefs about uncertainty and its implications, and involves the tendency to react negatively on emotional, cognitive and behavioural levels to uncertain situations and events*" (Buhr and Dugas, 2009). Intolerance of uncertainty is sometimes considered conceptually and empirically synonymous with anxiety – indeed, it is a core feature of GAD (Dugas et al., 1997, 2004). However, recent evidence suggests that intolerance of uncertainty is actually a transdiagnostic construct – in a heterogeneous group of patients with depressive and anxiety disorders, Boswell *et al.* found that intolerance of uncertainty significantly correlates with pre-treatment depressive and worry symptoms (Boswell et al., 2013). Enhanced intolerance of uncertainty has also been associated with increased vmPFC (BA10) activation to aversive CSs during fear extinction paradigms (Morriss et al., 2015).

Therefore, emergent evidence from several lines of work has made it apparent that vmPFC activity is linked to multiple aspects of negative emotion: fear extinction, fear regulation/stress recovery and intolerance of uncertainty. Furthermore, disrupted vmPFC activity has been directly linked to psychopathology typified by enhanced negative emotion: both anxiety disorders (Shin and Liberzon, 2010) and depressive disorders (Mayberg et al., 2005). However, whether over-activity within the vmPFC is causally linked to the enhanced negative affect characteristic of these disorders remains unknown.

From correlative human neuroimaging studies, there is a growing appreciation that over-activity in caudal regions of vmPFC – in particular sgACC/25 – may play a crucial role in negative emotion and its abnormal expression in both depression and anxiety (Alvarez et al., 2011; Hasler et al., 2007b; Mayberg et al., 2005; Phan et al., 2002). However, the causal role of this subregion remains unclear. Using the marmoset, we took a multi-dimensional approach to address causal links between over-activity in this region and negative emotion:

- Assessment of fear extinction using a fear conditioning/extinction paradigm with an ethologically relevant US (Snake Extinction);

[3] State anxiety refers to transient manifest feelings of insecurity, whereas trait anxiety is a stable personality characteristic reflecting the tendency to respond with state anxiety in anticipation of threatening situations.

- Assessment of fear learning and stress recovery during a discriminative aversive Pavlovian conditioning paradigm (Fear Discrimination); and
- Assessment of intolerance of uncertainty and anxiety using the human intruder (HI) test;

In so doing, we sought to derive a comprehensive account for the role of NHP sgACC/25 in anxiety- and fear-related behaviours relevant to mood and anxiety disorders. Owing to the acute, transient nature of these manipulations, impacts on behavioural and autonomic function would reflect a role of vmPFC subregions in state anxiety rather than trait anxiety.

As described in **Chapter 4**, the novel antidepressant ketamine successfully ameliorated symptoms of anticipatory anhedonia induced by sgACC/25 over-activation. Whilst a plethora of studies have demonstrated the efficacy of ketamine in mood disorder settings, far fewer studies have investigated ketamine's function in the context of anxiety disorders. The first study investigating the effects of ketamine on anxiety symptoms was carried out in 2017 – the effects of three subcutaneous doses (0.25, 0.5 and 1.0mg/kg) on treatment refractory GAD/SAD were assessed (Glue et al., 2017). Whilst minor improvements were observed at the lowest dose, 0.5 and 1mg/kg doses produced anxiolytic responses in ten of 12 patients – measurable within one hour of dosing and sustained for seven days. Subsequently, an uncontrolled open-label study assessed the safety, efficacy and tolerability of weekly/bi-weekly subcutaneous ketamine injections in the treatment of GAD and SAD (Glue et al., 2018). The authors reported that ketamine dosing was well tolerated, and patients experienced "*marked improvements in functionality and in their personal lives*." A randomised, placebo-controlled trial examining the effects of ketamine on SAD also observed promising beneficial effects on social anxiety scores (Taylor et al., 2018). We therefore sought to determine the ability of ketamine to reverse anxiogenic deficits – as measured by enhanced intolerance of uncertainty on the HI paradigm – associated with sgACC/25 over-activation (should there be evidence of any). Not only would this provide further insight into the neural basis of ketamine's action, but it would also serve as a direct comparison to the effects of ketamine on symptoms of anhedonia induced by the exact same manipulation.

5.3 METHODS

5.3.1 Subjects

Eight marmosets (three females, five males) took part in this study. These marmosets were Subjects 1-7 of cohort one and Subject 16 of cohort two, described in **2.1.1 SUBJECTS**. The marmosets were housed and cared for as described in **2.1.2 HOUSING**.

5.3.2 Surgical procedures

Eight marmosets underwent two surgical procedures prior to taking part in the study: one to implant a telemetric blood pressure probe and one to implant intracerebral cannulae targeting sgACC/25 and pgACC/32 (see **2.2 SURGICAL PROCEDURES**). PgACC/32 cannulae were not used in this study.

5.3.3 Behavioural testing apparatus and paradigms

5.3.3.1 Snake Extinction test

During the Snake Extinction test, animals were placed inside a Perspex carry box inside the testing chamber (described in **2.3 BEHAVIOURAL TESTING APPARATUS**). The white walls of the chamber had points onto which context panes (laminated sheets consisting of different patterns) could be attached. One wall consisted of a pane of switchable SmartGlass (smartglass International®, Dublin, Ireland). The opacity of the SmartGlass pane can be altered when voltage is applied, changing from opaque to transparent. When the SmartGlass became transparent, it revealed an additional section of the testing chamber. During acquisition of fear conditioning, this section contained a rubber snake on a mount. During habituation, extinction and extinction recall, this section contained a mount without a rubber snake.

A single block of the Snake Extinction test consisted of five sessions, run over five consecutive days (**FIGURE 5-2**). In the first two sessions, subjects were habituated to the context and the US-: this involved 12 x 5s presentations of the SmartGlass illuminating (but remaining opaque) with an ITI of 110-130s. In the third session of the block – acquisition – the CS was introduced. The CS was a 15s, 70dB auditory cue. The CS persisted for the 5s of the US to co-terminate with the US and the end of the trial. The first three CS presentations were paired with the US-. Following the first three presentations of CS/US-, the experimenter switched on the voltage supply to the SmartGlass pane: the US+ was 5s and consisted of the SmartGlass pane illuminating and becoming transparent, to reveal a chamber containing a rubber snake on a mount. There were then six pairings of the CS with the US+. During acquisition, CSs were presented with an ITI of 160-180s. In the fourth session – extinction – 20 CS/US- pairings were presented with an ITI of 60-80s to promote the extinction of conditioned fear. Infusions of saline vehicle or DHK were carried out

immediately prior to the extinction session to determine the effects of sgACC/25 over-activation on the extinction of conditioned fear. In the final, fifth session – extinction recall – 12 CS/US- pairings were presented with an ITI of 70-110s to test for recall of fear extinction.

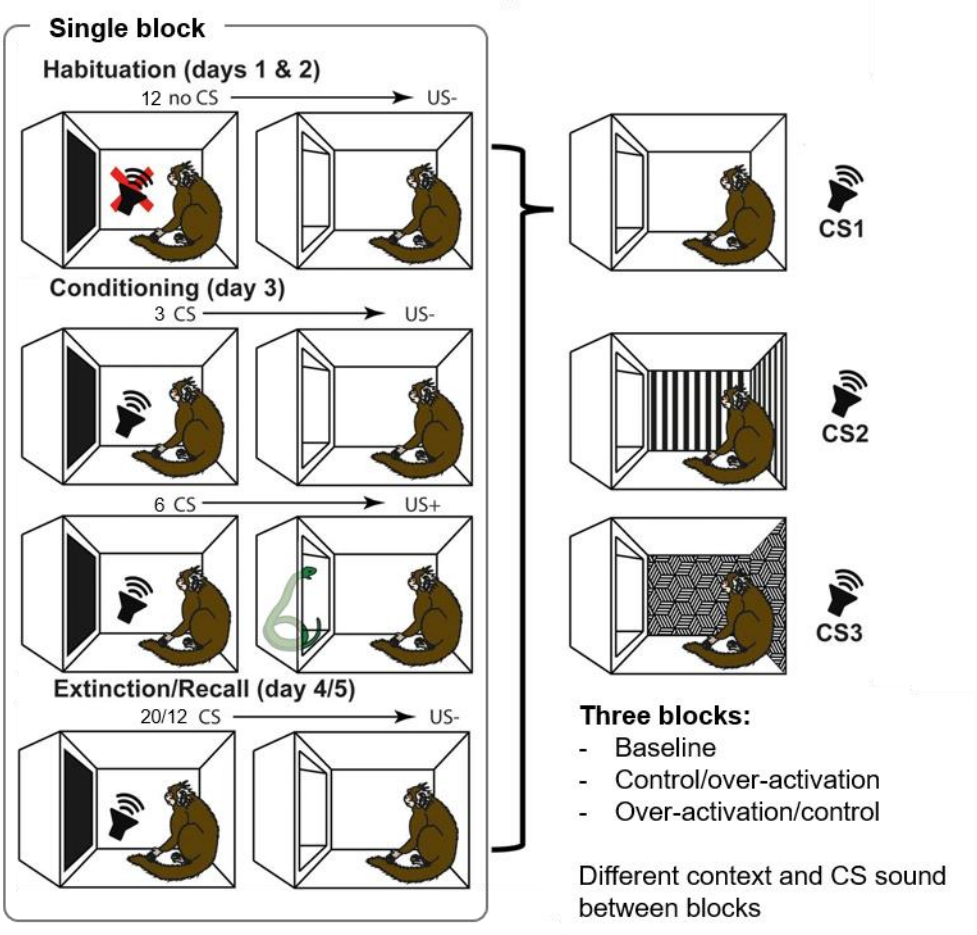


Figure 5-2 Snake Extinction testing paradigm. Figure adapted from Wallis *et al.*, 2017. In the Snake Extinction paradigm, a single block consists of five sessions spread over five consecutive days. The first two sessions consisted of habituation to the context and habituation to the SmartGlass being switched on (12 x US-) with no auditory cues. The mean MAP responses during habituation sessions were used to normalise the MAP responses in subsequent acquisition and extinction/recall sessions. On the third session, a novel auditory cue (to-be 'CS') was presented for three trials paired with 3 x US- (to habituate to the novel cue) and then this same cue was presented for six trials paired with 6 x US+ (presentations of the rubber snake, revealed as the SmartGlass became transparent) to become a CS. On the fourth and fifth sessions, marmosets were tested for extinction (20 x CS/US-) and extinction recall (12 x CS/US-) where the CS was presented in extinction. Each time a session block was repeated, the test apparatus was covered with distinctive patterned context panels to create a different context, and a different CS was used (shown right). Context, cues and context/cue combinations were counterbalanced across animals.

Blocks of five sessions were repeated three times within each subject. The three blocks included two control infusions of saline vehicle and one infusion of DHK. The first block (always saline) was used as a habituation block – the habituation sessions of this block were not comparable to the second and third blocks because the animal had never experienced an aversive US in the chamber prior to the first block. Therefore, the second and third blocks were the blocks used in data analysis, and saline vehicle and DHK infusions were counterbalanced within these blocks. There was a minimum of a one-week gap between each block. To minimise fear generalisation across blocks, patterned context panels were used to vary the context, and different sounds were used as the CS to distinguish each block as a new round of fear conditioning. Wall panels and CS sounds were counterbalanced across sessions.

5.3.3.2 Fear Discrimination test

During the Fear Discrimination test, animals were placed inside a Perspex carry box inside the testing chamber as above. The SmartGlass pane was not used during Fear Discrimination testing.

Marmosets were first exposed to two novel auditory cues (20s) and the cardiovascular arousal response (MAP) was measured. The cue that produced the smallest arousal response became the CS+ and the cue that produced the largest arousal response became the CS-. The animals were then trained on an aversive Pavlovian Fear Discrimination paradigm (**FIGURE 5-3**): the CS+ was associated with 30s of darkness, with 10s of 85dB white noise pseudo-randomly presented either in the first, second or third 10s window of the darkness (US+). The CS- was associated with a 0.5s 80dB neutral 2kHz tone (US-). The CS+ continued to play during the entire 30s period of the US+, whereas the CS- terminated prior to tone onset. ITIs were pseudo-randomly varied between 100-160s. Each session consisted of two to four trials with no more than one CS/US+ trial in a single session. See **TABLE 5-1** for the testing schedule. Infusions were always conducted on CS-/CS+/CS- sessions which lasted between 470-500s. This session structure was chosen for several reasons:

- The first CS- would measure if there were any abnormal responses simply to the first (neutral) auditory cue;
- Responses during the CS/US+ would measure if there were abnormally elevated responses to fear-predicting cues (CS+), fearful stimuli themselves (US+) and/or during the recovery following a fearful stimulus (post US+);
- The second CS- would measure if there were any abnormal responses to the neutral cue *after* presentation of an aversive stimulus; and

- Comparing responses between the first and second CS- would indicate whether there was an overall generalisation effect, or a generalisation effect specific to the pre- or post-CS/US+ period (or no generalisation at all).

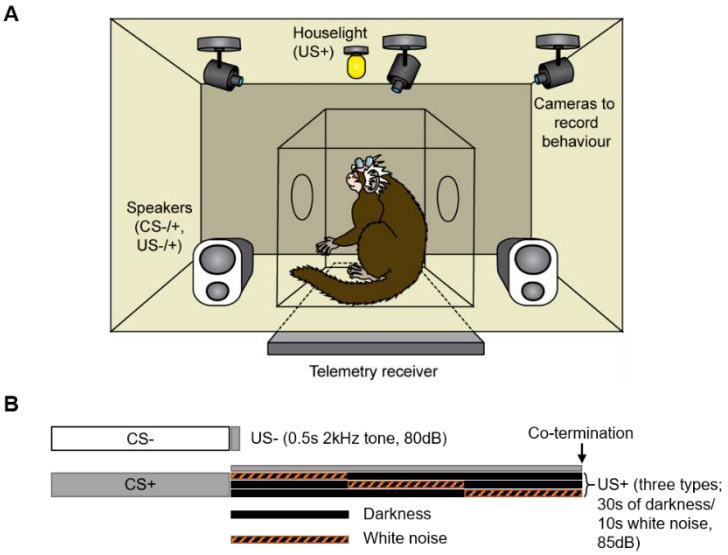


Figure 5-3 Fear Discrimination paradigm. **A** Schematic diagram of the Fear Discrimination apparatus. **B** Animals learnt to distinguish between two auditory CSs. The CS- predicted a 0.5s US-, consisting of a non-aversive 80dB 2kHz tone. The CS+ predicted a 30s US+, consisting of 30s of darkness with 10s of 85dB white noise unpredictably presented either in the first, middle or last 10s window. The CS+ co-terminated with the US+.

	Schedule 1		Schedule 2	
Week 1	Mon	-/-/-	Mon	-/-/+
	Tue	-/-	Tue	-/-/-
	Wed	+/-/-/-	Wed	-/-/+/-
	Thu	-/-/-	Thu	-/-
	Fri	-/+/-	Fri	-/+/-
Week 2	Mon	-/-/+/-	Mon	-/-/-
	Tue	+/-/-	Tue	+/-/-
	Wed	-/-	Wed	-/-
	Thu	-/+/-	Thu	-/+/-
	Fri	-/-/-	Fri	-/-/-

Table 5-1 Experimental testing schedule for Fear Discrimination. + represents CS+/US+; - represents CS-/US-. There were no more than five CS+/US+ presentations over a two-week period. Infusions were carried out on highlighted days.

5.3.3.3 Human Intruder (HI) test

The HI test was carried out in the marmosets' home cage. During the test, the animal was separated from its cage mate in the upper right quadrant (dimensions: 94 x 60 x 98cm) (**FIGURE 5-4A**). The upper right quadrant had a fixed configuration: a nest-box in the top right corner, an upper and lower perch, a front shelf, a middle shelf, a back shelf and a rope. The cage mate was placed in the lower left quadrant to minimise its vision of the human intruder and to reduce any disturbance to the test subject. A video camera mounted on a tripod was positioned approximately 100cm away from the cage front at an angle to maintain an adequate view of the entire upper right quadrant during the test. This was connected to a microphone positioned approximately 15cm away from the cage front, used to record vocalisations. During the test phase, the HI stood on a 20cm stool placed 40cm away from the front of the cage to maintain a fixed height and made eye contact with the test subject at all times.

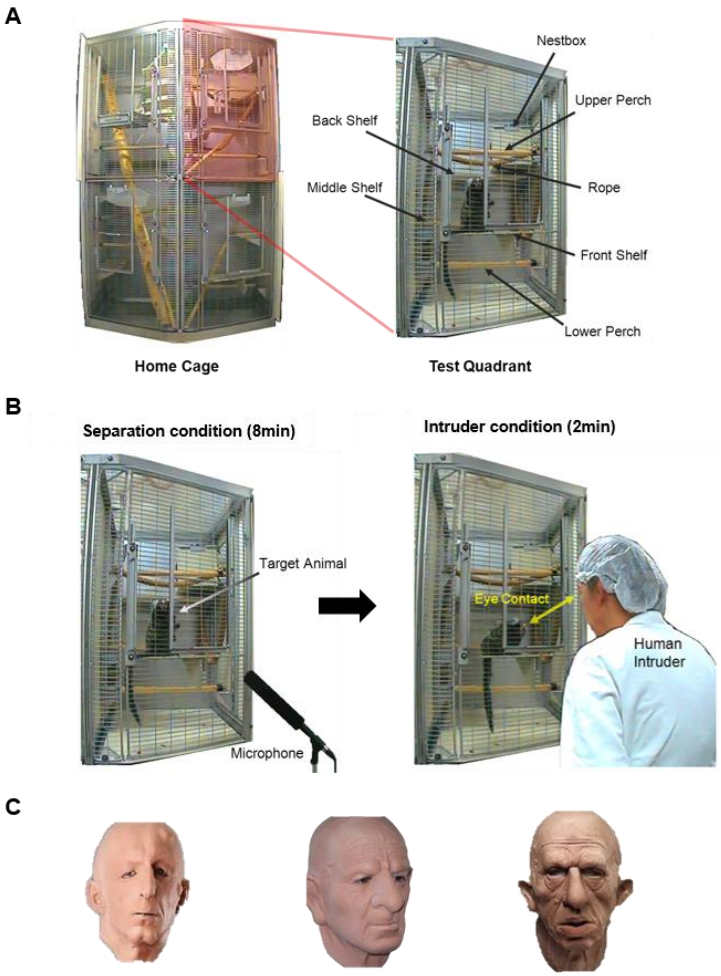


Figure 5-4 HI testing apparatus. Taken from Shiba, 2012 (PhD thesis). **A** The home cage is shown left, consisting of various environmental enrichment objects. Highlighted and shown right is the test quadrant in which the animal is separated during HI testing. The objects which remain in

place during HI testing are labelled. **B** The HI test lasted 15 minutes and consisted of three phases: an 8-minute separation condition without an HI; a 2-minute intruder condition; and finally, a 5-minute post-intruder condition (not shown). **C** Latex masks were used to disguise the HI, facilitating the use of a single individual in a repeated-measures design within single animals.

The HI test lasted 15 minutes in total (**FIGURE 5-4B**). The first 8 minutes constituted the 'separation' condition, in which the animal was recorded without the presence of the HI. This served as a habituation period. After the separation condition, the intruder entered the room and stood on the stool, beginning the two-minute 'intruder' condition. Throughout the intruder period, the HI maintained eye contact (whenever possible) with the test subject. The HI then left the room, and the subjects' behaviour was recorded for a further five minutes ('post-intruder' condition). At the end of the recording, the marmoset was undivided from the test quadrant and the recording equipment was removed.

The HI wore a white lab coat, blue lab gloves, blue trousers and one of set of realistic human masks (**FIGURE 5-4C**). The use of different masks disguised the HI as a novel intruder, facilitated a repeated-measures, within-subject design using a single individual as the intruder. The order of masks was counterbalanced between subjects.

The HI test was carried out twice per subject with a minimum of 10 days between consecutive tests. Infusions of either saline vehicle or DHK were carried out. In the first two subjects, the order of infusions was counterbalanced. When we observed a preliminary indication of an anxiogenic effect of DHK infusion (see below), subsequent subjects in the study received infusions of saline vehicle first, and DHK second. This was to mitigate against the confounding effects of possible habituation on the interpretation of DHK effects if saline control was given second (the control anxiety score could be lower because of habituation, rather than an anxiogenic effect of DHK). Subjects taking part in the ketamine study underwent a third HI test (always last) to over-activate sgACC/25 1 day after having received ketamine.

5.3.4 Drug treatments

Central and peripheral drug treatments were carried out as described in **2.4 DRUG TREATMENTS**. The pharmacological compounds used in experimental manipulations in this study were: 0.9% saline (vehicle control), DHK (an EAAT2 inhibitor) and ketamine (an NMDA receptor antagonist). For details of doses and pre-treatment times, see **TABLE 2-4**.

5.3.5 Salivary cortisol sampling

In the case of acquisition and extinction sessions of the Snake Extinction test, salivary cortisol samples were taken and processed as described in **2.5 SALIVARY CORTISOL**

SAMPLING. Specifically, samples were taken (i) during the (mock, in the case of acquisition) infusion ('pre') and (ii) after the acquisition/extinction session ('post' sample).

5.3.6 Data acquisition and preliminary analysis

For studies involving telemetric measurements, MAP and HR values were collected as described in **2.6.1 TELEMETRY DATA COLLECTION AND ANALYSIS**. MAP is used as the principal cardiovascular measure for two reasons: firstly, in both tests, cardiovascular conditioning was less variable with MAP values compared to HR values (Snake Extinction not shown, but for Fear Discrimination see **5.3.7.3**). Secondly, MAP was unaffected by DHK infusions in the neutral condition, whereas HR values are confounded by a baseline cardiovascular effect (**Chapter 3**).

5.3.6.1 Snake Extinction test

The mean MAP values during the 15s CS and 5s US period were calculated. MAP values were averaged in pairs (referred to as CS pairs, as in (Sierra-Mercado et al., 2011)) and normalised to the mean MAP response across the two habituation sessions within the same block (which reflected the MAP arousal to the context prior to acquisition). CS directed MAP responses were also calculated as $MAP_{CS} – MAP_{baseline}$ (15s pre-CS periods) and averaged across CS pairs during acquisition. CS directed responses are CS specific, and so reflect cue-based (as opposed to context-based) conditioning. To examine the profile of the cardiovascular responses across the entire acquisition, extinction and extinction recall sessions, MAP values were binned into 1s intervals.

Behaviour was scored offline from video-recordings of the session. The behaviour scored was vigilant scanning (VS) – attentive scanning of the surroundings accompanied by a tense body posture (Agustín-Pavón et al., 2012; Mikheenko et al., 2010; Wallis et al., 2017). Absolute and CS directed VS measures were calculated and averaged across CS pairs as above but were not normalised to habituation sessions as animals did not scan prior to acquisition.

5.3.6.2 Fear Discrimination test

The mean MAP during the 20s BL, CS and 30s US+ period was calculated; CS directed, US directed ($MAP_{US} – MAP_{CS}$) and absolute responses are reported. The behaviour scored was VS – both absolute and CS directed VS are reported. To examine the profile of the cardiovascular response across the entire CS-/CS+/CS- session, MAP values were binned into 1s intervals. The ten, 1s bins period following termination of the US+ was defined as the recovery (R) period – this period is of *a priori* interest as impaired stress recovery is a key feature of psychiatric disorders (Burke et al., 2005).

5.3.6.3 HI test

A behavioural analysis program (JWatcher v1.0, UCLA and Macquarie University) was used to score behaviour during the two-minute intruder phase. The behavioural measures were:

- **Distance measures**. The proportion of time spent in each depth and height zone (**FIGURE 5-5A, B**) was scored. The average height/depth was calculated by multiplying the proportion of time spent in each zone by the distance of the middle point of that zone from the floor/front of the quadrant.
- **Locomotion**. Locomotion is defined as translational movement in which all four limbs change location. The percentage of time spent in locomotion was scored.
- **Head and body bobs**. Head and body bobs are marmoset behaviours indicative of anxiety (Agustín-Pavón et al., 2012; Carey et al., 1992; Santangelo et al., 2016). The number of head and body bobs was scored.
- **Vocalisations**. When confronted with a human intruder, marmosets exhibit a repertoire of vocal responses – these include tsik, tsik-egg, tse, tse-egg and egg calls, separated based on differences in duration and frequency range (**FIGURE 5-6**) (Bezerra and Souto, 2008). Audio editing software (Audacity v2.2.2, https://sourceforge.net/projects/audacity/) was used to extract audio from the video recordings which was converted into a waveform (Syrinx, v2.6h).

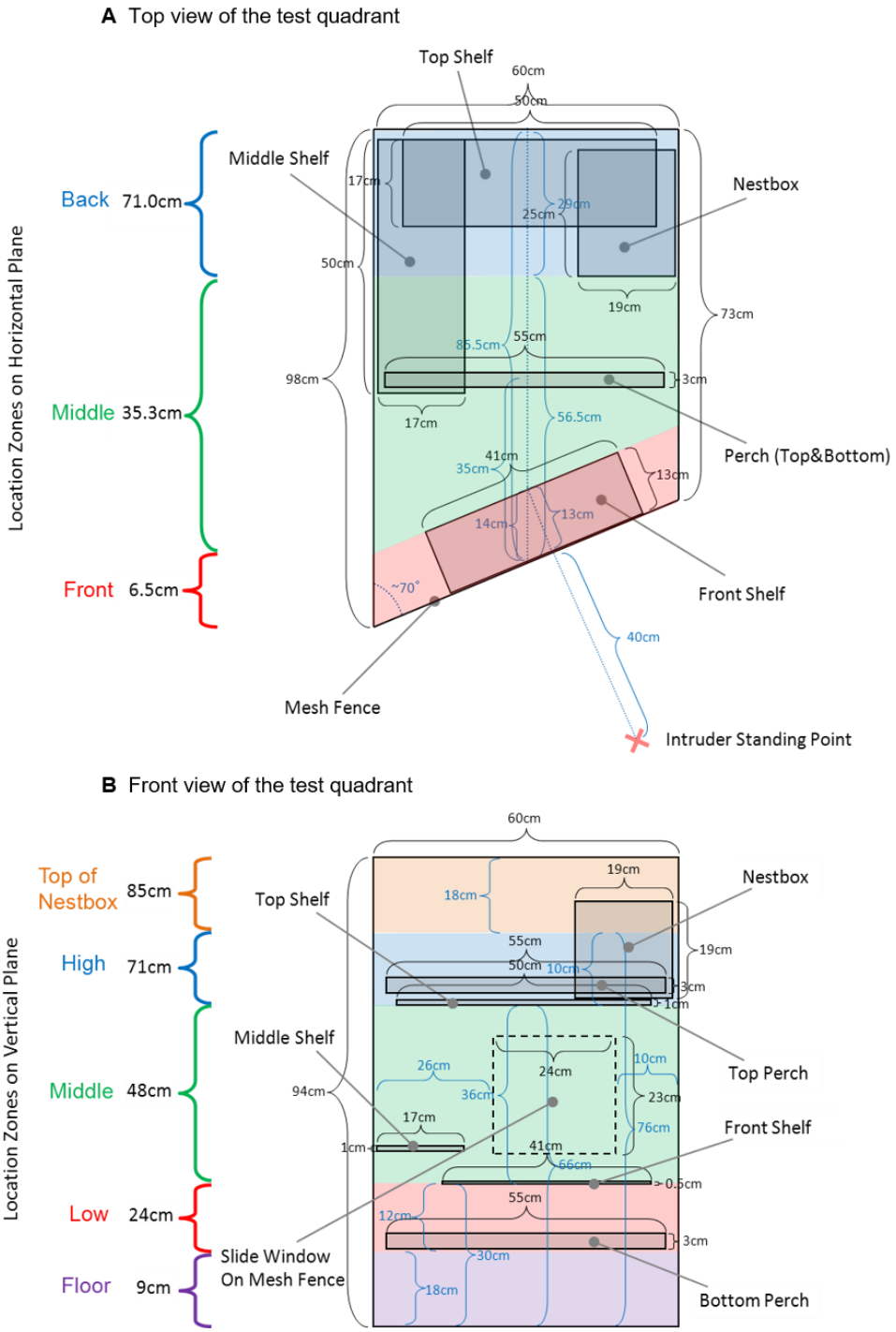


Figure 5-5 Dimensions of HI test quadrant. Taken from Shiba, 2012 (PhD thesis). **A** Top view and **B** front view of the test quadrant with the dimensions of all objects. The location zones are highlighted in different colours, and the locations of their respective midpoints in the horizontal/vertical planes are shown left, in cm.

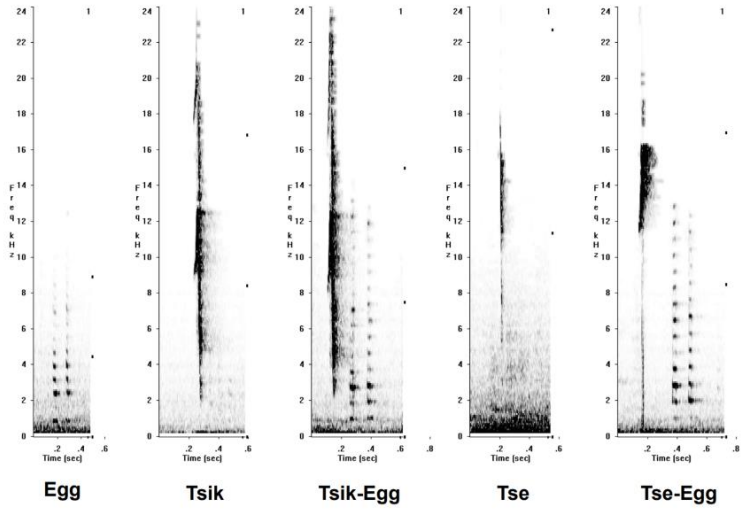


Figure 5-6 Vocalisations made during the HI test. Taken from Santangelo *et al.*, 2016. The typical bandwidth and frequency patterns of the five calls scored are shown, visualised using Syrinx (v2.6h).

5.3.7 Statistical analysis

Data were inputted into GraphPad Prism v8.00.178 for Windows (GraphPad Software, La Jolla, CA) for statistical analysis. Significance was set at α=0.05 in all cases. In all ANOVAs, multiple comparisons were corrected for using Sidak's multiple comparisons test.

5.3.7.1 Snake Extinction test: control condition

5.3.7.1.1 Acquisition

To determine if animals had successfully acquired the fear association under control conditions, a two-tailed paired *t*-test was conducted comparing normalised MAP and absolute VS values between the final pre-acquisition CS pair (3-4 [CS4 still being 'pre'-snake]) and the final acquisition (post-acquisition) CS pair (8-9). The same analysis was carried out on CS directed MAP/VS values.

5.3.7.1.2 Extinction and Extinction Recall

To determine if animals successfully extinguished the fear association across 20 CSs presented in extinction, two analyses were carried out. First, best-fit lines were generated for each animal's MAP/VS extinction profile using linear regression. The gradients of these best-fit lines were compared to a hypothetical value of 0 (a flat line) using a one-sample *t*-test. Second, the MAP/VS arousal responses were compared between the first CS pair (1-2) and last CS pair (19-20) using a two-tailed paired *t*-test, to determine if they were significantly different from one another. Extinction recall MAP and VS responses were plotted, and the

gradients of MAP/VS best-fit lines were compared to a hypothetical value of 0 using a one-sample *t*-test (to see if animals continued to extinguish on this day, or if there was successful recall and therefore no further extinction).

5.3.7.2 Snake Extinction test: drug manipulations

5.3.7.2.1 Acquisition

To determine if there was any difference in acquisition profiles during the to-be-control and to-be-over-activation blocks, the normalised MAP and absolute VS values of the final pre-acquisition CS pair were compared to the final acquisition (post-acquisition) CS pair across both blocks using a two-way repeated measures ANOVA of the form $M_2 \times P_2$: *M* is a factor with two levels (manipulation) and *P* is a factor with two levels (CS pair; pre- *vs.* post-acquisition).

'Post':'pre' salivary cortisol ratios were calculated for each acquisition block (three subjects, two blocks each for a total of six blocks). These ratios were compared to a hypothetical value of 1.0 (no change) using a one-sample *t*-test, to determine if acquisition significantly elevated salivary cortisol.

5.3.7.2.2 Extinction and Extinction Recall

Normalised MAP and absolute VS values during the CS periods of the extinction phase were analysed using a two-way repeated measures ANOVA of the form $M_2 \times P_{10}$ where *M* is a factor with two levels (manipulation type) and *P* is a factor with ten levels (CS pair). Normalised MAP and VS values during the extinction recall phase were analysed using a two-way repeated measures ANOVA of the form $M_2 \times P_6$ where *M* is a factor with two levels (manipulation type) and *P* is a factor with six levels (CS pair).

Further analysis sought to determine whether there was a non-CS specific (contextual) effect of sgACC/25 over-activation on cardiovascular MAP or behavioural VS values during extinction/extinction recall sessions. For cardiovascular arousal, this was done by comparing MAP profiles across the entire extinction/extinction recall session (excluding the first minute). To statistically compare the MAP profiles, an ANOVA was performed with R version 3.4.1 using the lme4 package (Bates et al., 2014) for linear mixed-effects modelling, with statistical tests from the lmerTest package (Kuznetsova et al., 2016) using type III sums of squares with the Satterthwaite approximation for degrees of freedom, here reported to the nearest integer. Factors included fixed effect factors: treatment (saline control, or over-activation with DHK) and time; and a random effect factor of subject (*i.e*. the individual marmosets) to account for inter-individual differences between animals. To determine if there was a contextual effect on behavioural arousal, baseline (pre-CS) VS values were averaged across

extinction/extinction recall sessions and compared between control and over-activation conditions using a two-tailed paired *t*-test.

5.3.7.3 Fear Discrimination test: illustrating discrimination

To illustrate successful discrimination between CS+ and CS-, a one-way repeated measures ANOVA was carried out comparing MAP and VS responses to the first CS-, CS+ and second CS- on the CS-/CS+/CS- session immediately prior to infusions. Additionally, a two-tailed paired *t*-test was carried out to compare CS+ responses to mean CS- responses. US directed (US minus CS) MAP responses were compared to a hypothetical value of 0 (no change compared to CS period) using a one-sample *t*-test.

5.3.7.4 Fear Discrimination test: drug manipulations

For drug manipulation sessions, it was first determined if there were any differences in CS directed MAP/VS responses. A two-way repeated-measures ANOVA of the form $M_2 \times C_3$ was carried out where *M* is a factor with two levels (manipulation type) and *C* is a factor with three levels (CS type: first CS-, CS+ or second CS-). A two-tailed paired *t*-test was carried out to compare US directed responses during the US+ period.

Further analysis sought to determine whether there was a non-CS specific (contextual) effect of sgACC/25 over-activation on absolute MAP or VS values. Absolute MAP values during the baseline and CS periods were compared across infusion type using separate two-way repeated-measures ANOVAs of the form $M_2 \times B_3/C_3$ where *M* is a factor with two levels (manipulation type) and *B/C* is a factor with three levels (baseline/CS type: first, second or third). Absolute VS values were statistically tested in an identical way. The MAP profile across the entire session (excluding the first minute) was also assessed and statistically compared using an ANOVA was performed with R version 3.4.1 using the lme4 package (Bates et al., 2014) for linear mixed-effects modelling, with statistical tests from the lmerTest package (Kuznetsova et al., 2016) using type III sums of squares with the Satterthwaite approximation for degrees of freedom, here reported to the nearest integer. Factors included fixed effect factors: treatment (saline control, or over-activation with DHK) and time, and random effect factors: subject (*i.e*. the individual marmosets) to account for inter-individual differences between animals.

During the 10s recovery period after the US+, a ratio was calculated comparing the MAP value in each 1s bin to the MAP value in the final 1s bin of the US+ period. Ratio values for control and over-activation conditions were compared using a two-way repeated measures ANOVA of the form $M_2 \times T_{10}$ where *M* is a factor with two levels (manipulation type) and *T* is a factor with ten levels (ten 1s time bins).

5.3.7.5 HI test

An exploratory factor analysis (EFA) with a principal axis factoring extraction method has been performed on HI Test scores from sessions carried out as part of a screening procedure on 171 marmosets from the colony (unpublished data). This model predicts the extent to which the different behaviours in the human intruder test are driven by an underlying latent variable. Initial runs of the exploratory factor analysis included: percentage of time spent at front (TSAF) and time spent at the back (TSAB) of the cage, average height, percentage of time spent in locomotion, number of bobs, egg calls, tsik call, tsik-egg calls, tse calls, and tse-egg calls. Instead of average depth, the TSAF/TSAB were used, as these measures reflect approach and avoidance movements respectively and appear more sensitive to changes in anxiety levels of the marmosets (unpublished findings). Tse calls were removed from the exploratory factor analysis as its measure of sampling adequacy was below the standard of 0.5 defined in Field, 2009 (MSA = 0.424) leaving a total of nine variables. The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy for the final model verified the sampling adequacy for the analysis, KMO = 0.82 ('great' according to (Hutcheson and Sofroniou, 1999)). Bartlett's test of sphericity was significant ($\chi 2$ [36] = 460.84, $p<0.001$), indicating that correlations between items were sufficiently large for a factor analysis. Due to the low level of communalities after extraction, the scree plot was consulted to decide the number of factors to extract instead of using Kaiser's criterion (Field, 2013). Only 1 factor was extracted based on the point of inflection on the Scree plot (**FIGURE 5-7A**). This factor accounted for 39.7% of the variance. There were 16 (44.0%) nonredundant residuals with absolute values greater than 0.05, below the recommended value of 50%, reflecting that the one factor model is a good fitting model. The pattern in which the items cluster on this factor suggest that this factor represents the animal's anxiety (**FIGURE 5-7B**) with high scores reflecting increased depth and height in the cage, together with increases in vigilant bobbing and egg calls.

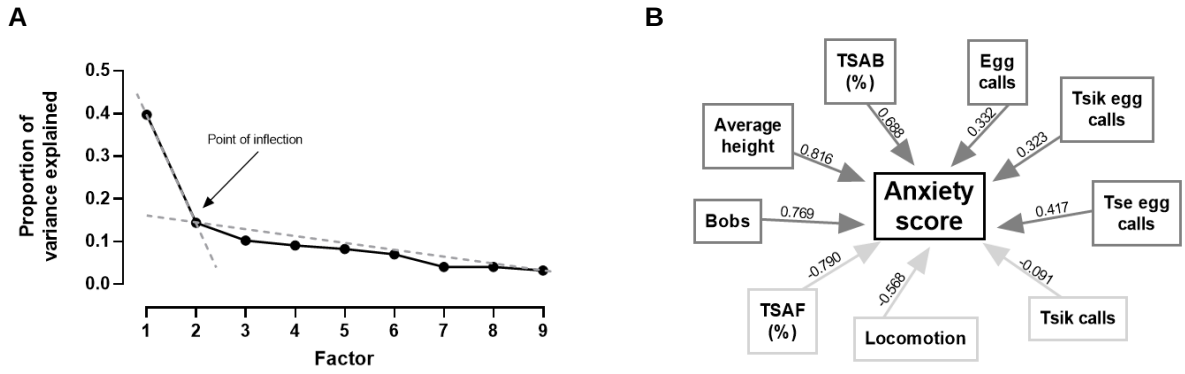


Figure 5-7 The use of an exploratory factor analysis (EFA) to extract latent variables explaining variance in behaviour. **A** An EFA was carried out as part of a screening procedure on 171 marmosets in the University of Cambridge Marmoset Breeding Colony to predict the extent to which marmosets' responses to an HI are driven by underlying latent factors. In total, nine factors were extracted. The point of inflection on the Scree plot suggests a one factor model is sufficient, accounting for 39.7% of the total variance in responding across the colony. **B** The loading of each behavioural measure onto the factor. Each measure loads positively (dark grey) or negatively (light grey) with different weights indicated by values above the arrows. The pattern in which behaviours cluster onto the factor suggest that the factor represents marmosets' anxiety towards the HI (anxiety score): a higher score is associated with increased depth from the cage front, increased height and increased vigilance in the form of head bobbing and egg vocalisations.

A two-tailed paired *t*-test was conducted on the EFA-derived anxiety scores for saline control *vs.* sgACC/25 over-activation to determine if sgACC/25 over-activation had any effect on anxiety levels during periods of uncertainty. Individual measures were compared using individual two-tailed paired *t*-tests to determine which behaviours were driving the change in anxiety scores.

5.3.7.6 Ketamine study (HI test)

The statistical analysis of behavioural data to generate the anxiety scores was performed using EFA, as described above. A one-way repeated-measures ANOVA was carried out to compare the effects of control, over-activation and (over-activation + ketamine) manipulations on anxiety scores.

5.3.8 Post-mortem histological processing

Of the cohort of eight animals used in this study, five are still alive and undergoing ^{18}F-FDG PET akin to that described in the previous chapter. For the three animals where post-mortem data are available, the brain sections were prepared and visualised as described in **2.8 POST-MORTEM ASSESSMENT OF CANNULA PLACEMENT**.

5.4 RESULTS

5.4.1 Post-mortem assessment of cannula placement

Histological analysis revealed that of the three animals assessed, all had cannulae successfully targeting sgACC/25 (**FIGURE 5-8**). The other animals constituting this cohort are still alive.

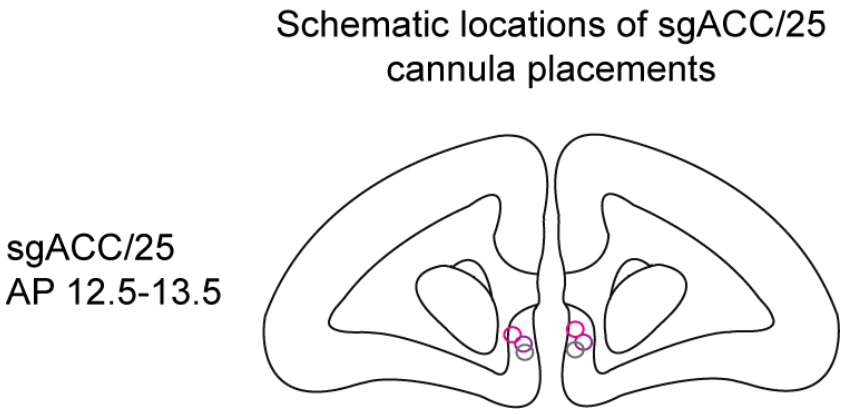


Figure 5-8 Cannula placements. Location of sgACC/25 cannulae for the three animals where post-mortem placements are available.

5.4.2 Animals show both cue- and context-directed conditioning following Snake Extinction acquisition sessions under control conditions

On the acquisition session of control Snake Extinction blocks, animals successfully acquired conditioned fear as indicated by an increase in normalised MAP and absolute VS responses measured in the post-acquisition CS pair compared to the pre-acquisition CS pair (**FIGURE 5-9A, B**). CS directed acquisition of cardiovascular MAP arousal was variable: specifically, there was no significant difference between pre- and post-acquisition phases for CS directed MAP values (**FIGURE 5-9C**). This suggests that increases in MAP arousal during the CS period are predominantly driven by contextual associations. By contrast, behavioural VS responses were CS specific, as there was a significant difference in CS directed VS behaviour between pre- and post-acquisition phases (**FIGURE 5-9D**).

To further evidence contextual cardiovascular/behavioural conditioning effects in addition to cue-specific learning, (i) absolute MAP responses were plotted across the entire session to qualitatively determine if there was a systematic elevation in MAP following snake presentation; and (ii) mean baseline (15s pre-CS period) VS responses were compared between pre- and post-acquisition trials. In the baseline period, no auditory cues are presented and so changes in behavioural arousal during this period must reflect an increased response to the context. When MAP arousal was plotted across the entire acquisition session, it was apparent that there was a systematic increase in MAP levels

following snake exposure which was not specific to any discernible CS period (**FIGURE 5-9E**). Behaviourally, baseline VS responses increased in two out of three animals and in the third animal remained at zero; across all three subjects, this increase was not significant (**FIGURE 5-9F**).

In sum, these data suggest that the principal measures extracted from the Snake Extinction study – normalised MAP and absolute VS values during CS pair periods – reflect a combination of context- (MAP and to some extent, VS) and cue- (VS) driven responding.

5.4.3 Animals show extinction and recall of extinction under control conditions

Under control conditions, animals exhibited successful extinction across the ten CS pairs presented in extinction, evidenced by a significantly negative gradient (determined from best-fit lines of individual subjects) for normalised MAP (**FIGURE 5-9G**) and absolute VS (**FIGURE 5-9H**) responses across the session. Successful extinction was also evident as a significant difference in normalised MAP (**FIGURE 5-9G**) and absolute VS (**FIGURE 5-9H**) values between the first CS pair (CS 1-2) and last CS pair (CS 19-20) presented during extinction. On the following extinction recall day, the gradient of best fit lines did not significantly differ from 0 for either MAP arousal (**FIGURE 5-9I**) or VS arousal (**FIGURE 5-9J**) indicating that animals successfully recalled extinction and no further extinction took place.

5.4.4 Salivary cortisol levels are higher following acquisition

In addition to the behavioural and autonomic measures described above, salivary cortisol samples were taken before and after acquisition for both to-be-control and to-be-over-activation blocks. These samples indicated that ‘post’-acquisition levels of cortisol were significantly higher than ‘pre’-acquisition levels of cortisol as indexed by a ‘post:‘pre’ ratio significantly greater than 1.0 (**FIGURE 5-9K**). Therefore, the physiological responses to snake presentation are not limited to the cardiovascular domain, but additionally include elevated activity in the HPA axis as indexed by salivary cortisol. The sensitivity of the paradigm to changes in HPA axis activity meant we could go on to assess cortisol dynamics on day of extinction, comparing control and over-activation conditions (see below).

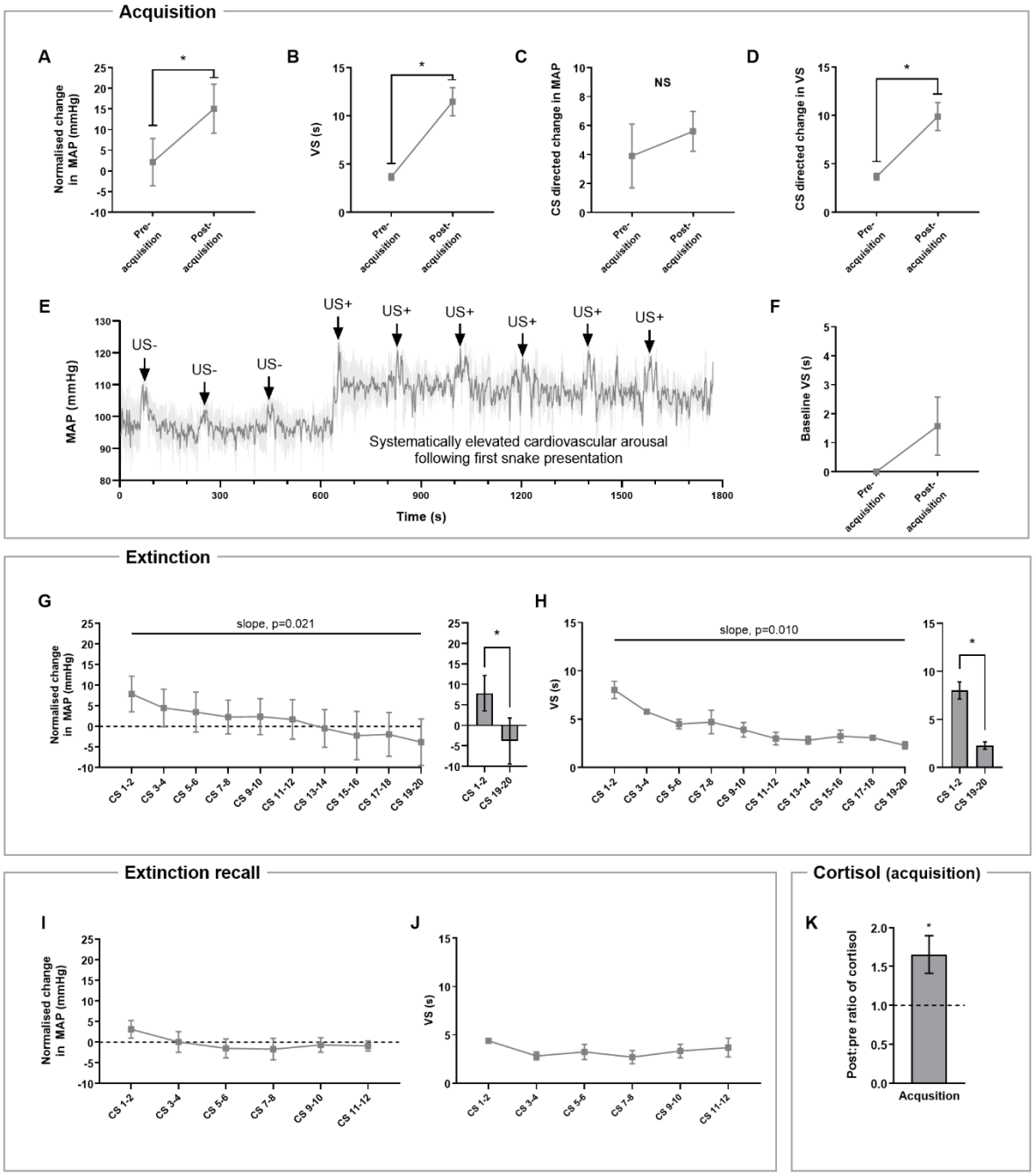


Figure 5-9 Features of acquisition, extinction and extinction recall under control conditions in the Snake Extinction paradigm. Relevant graphs show mean ± SEM. N=3. **A** Marmosets successfully acquired a fear association during acquisition, as indicated by a significant difference in normalised MAP response between the final pre-acquisition and post-acquisition CS pair (two-tailed paired *t*-test, p=0.021). **B** Successful acquisition was also evident in absolute VS responses (two-tailed paired *t*-test, p=0.036). **C** CS directed (cue specific) MAP arousal was more variable – there was no significant difference between pre- and post-acquisition CS pairs (two-tailed paired *t*-test, p=0.641). This indicates that MAP conditioning was predominantly context-directed. **D** There was reliable CS directed VS conditioning (two-tailed paired *t*-test, p=0.039), suggesting that behavioural arousal was CS-specific. **E** Further evidence for contextual learning is evident when MAP

responses were plotted across the acquisition session: following snake presentation, MAP arousal was systematically elevated in a manner not restricted to CS periods. The observable peaks represent exposures to the US (indicated with arrows). **F** Behaviourally, two out of three animals showed elevated levels of VS during baseline periods (and one showed no change), suggestive of a contextual behavioural response (although this increase was not significant; two-tailed paired *t*-test, p=0.258). In sum, these data suggest a mixed pattern of cue- (VS) and context- (MAP, VS) conditioning. **G** On the subsequent day, animals exhibited successful extinction of MAP arousal, as evidenced by significantly negative extinction gradients (one-sample *t*-test compared to 0, p=0.021), together with a significant decrease in MAP arousal during the first (CS 1-2) *vs.* last (CS 19-20) CS pair presented in extinction (two-tailed paired *t*-test, p=0.020). **F** Successful extinction was also evidenced in VS arousal – gradients were significantly negative (one-sample *t*-test compared to 0, p=0.010) and there was a significant difference between the first and last CS pair (two-tailed paired *t*-test, p=0.027). **I** Profile of MAP arousal responses during extinction recall. The gradient of individual best fit lines were not significantly different from 0 (one-sample *t*-test compared to 0, p=0.199) indicating that no further extinction of MAP arousal took place on the extinction recall day. **J** Profile of VS arousal responses during extinction recall. The gradient of individual best fit lines was not significantly different from 0 (one-sample *t*-test compared to 0, p=0.631) indicating that no further extinction of behavioural arousal took place on the extinction recall day. **K** 'Post':'pre' ratios of salivary cortisol samples showed that salivary cortisol levels were significantly higher post-acquisition (one-sample *t*-test compared to 1.0, p=0.043), suggesting that this behavioural session is associated with an endocrine response.

5.4.5 SgACC/25 over-activation increases cardiovascular and behavioural arousal during fear extinction, which remain elevated on the following extinction recall day

5.4.5.1 Acquisition

There was no difference in the level of cardiovascular (**FIGURE 5-10A**) or behavioural (**FIGURE 5-10B**) acquisition attained during to-be-control *vs.* to-be-over-activation blocks.

5.4.5.2 Extinction and Extinction recall

Infusions of saline vehicle (control) or DHK (over-activation) into sgACC/25 were carried out on the following day, immediately prior to the extinction session. Whilst over-activation of sgACC/25 had no effect on the rate of MAP extinction, it significantly, systematically increased MAP arousal during the CS periods (**FIGURE 5-10C**). The same effect was observed on VS behaviour: the rate of VS extinction was unchanged, but levels of VS in CS periods were significantly higher following sgACC/25 over-activation (**FIGURE 5-10D**). On the following extinction recall day, MAP and VS responses were again significantly higher during CS periods for animals which had undergone sgACC/25 over-activation the day before (**FIGURE 5-10E, F**). Furthermore, on extinction recall days, the manipulation × CS pair

interaction term showed a trend towards significance for MAP (p=0.054) and was significant for VS values (p=0.049), suggesting that extinction recall profiles were steeper for over-activation *vs.* control conditions. In the case of over-activation, it appears that animals continue to extinguish their responding over the following day.

To determine if the increases in CS period arousal were contributed to by contextual effects of sgACC/25 over-activation, whole-session MAP responses and baseline VS behaviour were assessed for both extinction and extinction recall sessions. During extinction sessions, over-activation resulted in significantly elevated MAP arousal, the degree of which decreased over the session (indicated by a significant manipulation x time interaction across the entire session, **FIGURE 5-10G**). Increased baseline VS behaviour was observed in all three animals (although owing to inter-individual variability in magnitude, this increase was not significant, p=0.187) (**FIGURE 5-10H**). These data suggest that sgACC/25 over-activation has a general effect to increase arousal in the aversive context. Given that there was no evidence for a difference in the rate of extinction within the CS periods (**FIGURE 5-10C**), the reduction in the magnitude of MAP arousal increase across the entire session may relate to the DHK effect slowly wearing-off over the 30 minute session (as DHK effects have previously been shown to last 15-30 minutes, (John et al., 2012)) rather than an increase in the rate of extinction. The significant contextual effects on cardiovascular arousal were also apparent during extinction recall, manifesting as a significant manipulation x time interaction for MAP values plotted across the entire session (**FIGURE 5-10I**). Given that no infusion happened on this day, the different slope cannot reflect an effect of the drug wearing off: instead, the interaction term suggests that over-activation the day before meant animals continued to extinguish their elevated arousal on the subsequent recall day. Increases in baseline VS behaviour were also apparent in all three animals on extinction recall (although again not significant, p=0.078) (**FIGURE 5-10J**). Collectively, these data support enhanced context-associated arousal following sgACC/25 over-activation – most consistently in the cardiovascular domain, but potentially exhibited in the behavioural domain too.

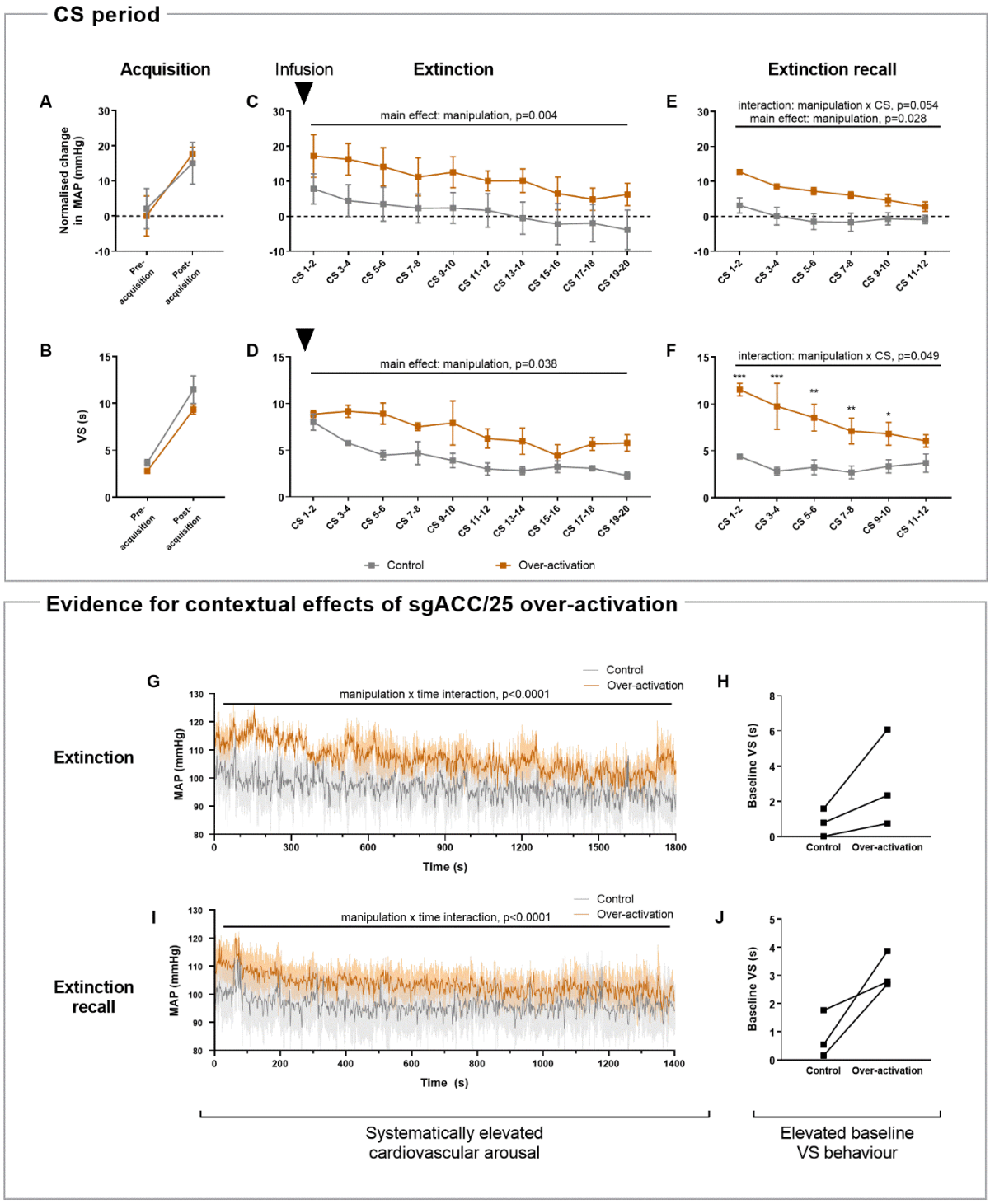


Figure 5-10 SgACC/25 over-activation increases cardiovascular and behavioural arousal during fear extinction, which remain elevated on the following extinction recall day. Relevant graphs show mean ± SEM. N=3. **A** There was no significant difference in acquisition of MAP responses across control and sgACC/25 over-activation blocks (manipulation × CS pair: $F<1$, NS; main effect of manipulation: $F<1$, NS). **B** There was no significant difference in acquisition of VS responses across control and sgACC/25 over-activation blocks (manipulation × CS pair: $F_{1,2}=1.59$ p=0.334; main effect of manipulation: $F_{1,2}=4.26$, p=0.175). **C** SgACC/25 over-activation 10 minutes prior to extinction did not affect the rate of MAP extinction (manipulation × CS pair: $F<1$, NS), but

systematically enhanced cardiovascular arousal measured across CS pairs (main effect of manipulation: $F_{1,2}$=239, p=0.004). **D** SgACC/25 over-activation did not affect the rate of behavioural extinction (manipulation × CS pair: $F_{9,18}$=1.26, p=0.323) but systematically enhanced VS behaviour measured across CS pairs (main effect of manipulation: $F_{1,2}$=24.8, p=0.038). **E** On the subsequent extinction recall day, the manipulation × CS pair interaction for MAP arousal showed a trend towards significance ($F_{5,10}$=3.24, p=0.054) suggesting that the two recall lines had different gradients and that animals continued to extinguish their MAP arousal during extinction recall (the main effect of manipulation was significant: $F_{1,2}$=34.5, p=0.028). **F** For VS behaviour, the gradient of extinction recall was different (manipulation × CS pair: $F_{5,10}$=4.41, p=0.049) with levels of VS differing most in early CSs (Sidak's multiple comparisons test: pairs one and two, p<0.001) but reaching similar levels by the final CS pair (p=0.244) suggesting that animals continued to extinguish their behaviour during extinction recall. **G** SgACC/25 over-activation systematically elevated cardiovascular arousal during extinction in a fashion which decreased over time as indicated by a significant manipulation x time interaction ($F_{1,10671}$=409, p<0.0001; main effect of manipulation: $F_{1,2}$=6.96, p=0.118), suggestive of a contextual effect. Given the lack of interaction effect measured during the CS period in isolation (**C**), this interaction may reflect an effect of the drug wearing-off across the ~30-minute session rather than differences in the rate of extinction. **H** In all three animals, baseline VS increased following over-activation, although this was not significant (two-tailed paired *t*-test, p=0.187). **I** SgACC/25 over-activation systematically elevated cardiovascular arousal during extinction recall in a fashion which decreased over time as indicated by a significant manipulation x time interaction ($F_{1,8318}$=329, p<0.0001; main effect of manipulation: $F_{1,2}$=7.10, p=0.116). There was no infusion this day (and this effect cannot therefore be an effect of drug wearing-off), therefore these data support the suggestion that animals which received sgACC/25 over-activation on the previous extinction day continued to extinguish their arousal over extinction recall. **J** In all three animals, baseline VS was increased on the extinction recall day, although this increase was not significant (two-tailed paired *t*-test, p=0.078).

5.4.6 SgACC/25 over-activation elevated salivary cortisol concentrations following extinction

Salivary cortisol samples were acquired pre- and post-session on the extinction day. The ratios of 'post':'pre' salivary cortisol were significantly higher following over-activation compared to control infusions, and effect observed in all three animals (**FIGURE 5-11**) indicating that whilst sgACC/25 does not affect cortisol levels in neutral conditions (**Chapter 3**), it appears to elevate HPA axis activity in aversive contexts. Note that salivary cortisol levels are but one measure of HPA axis output, and levels of cortisol in the saliva are determined by many factors (Ash et al., 2018). Therefore, further work is needed to determine whether this reflects a causal influence of sgACC/25 over-activity on HPA axis activity, or an indirect effect.

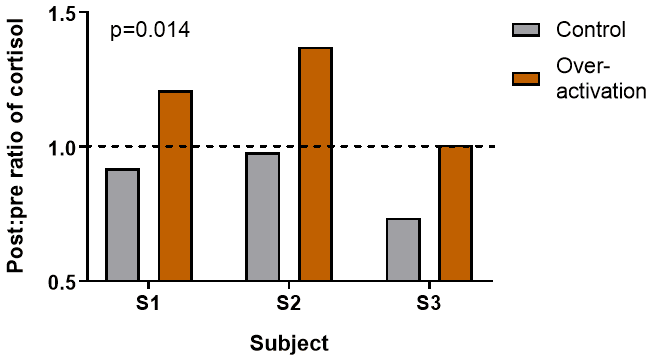


Figure 5-11 Ratio of 'post':'pre' salivary cortisol levels during extinction days under control and over-activation conditions. Following over-activation of sgACC/25, the ratio of 'post':'pre' cortisol was significantly higher across all three animals (two-tailed paired *t*-test, p=0.014).

5.4.7 Animals successfully acquired differential arousal responses to the CS+ and CS- on the Fear Discrimination paradigm

On the Fear Discrimination paradigm, animals were trained to distinguish between two auditory cues – a CS+ which predicted the presentation of 30s of white noise (85dB) and darkness (US+), and a CS- which predicted the presentation of a neutral 0.5s 2kHz tone (80dB) (see **FIGURE 5-3**). Sessions containing CS-/CS+/CS- were assessed for the experimental purposes outlined in **5.3**.

Animals successfully acquired cardiovascular discrimination, as evidenced by an increase in CS directed MAP responses to the CS+ but not the first/last CS- in CS-/CS+/CS- sessions immediately prior to infusions (**FIGURE 5-12A**). HR conditioning was more variable – whilst there was a trend towards discrimination, it was not significant (p=0.056; **FIGURE 5-12B**). During the US period, animals exhibited significant increases in MAP in response to the US+, over-and-above the rise observed during the CS+ (US directed, **FIGURE 5-12C**). Animals also successfully acquired behavioural discrimination, as evidenced by an increase in CS directed VS responses to the CS+ but not the first/last CS- in CS-/CS+/CS- sessions immediately prior infusions (**FIGURE 5-12D**).

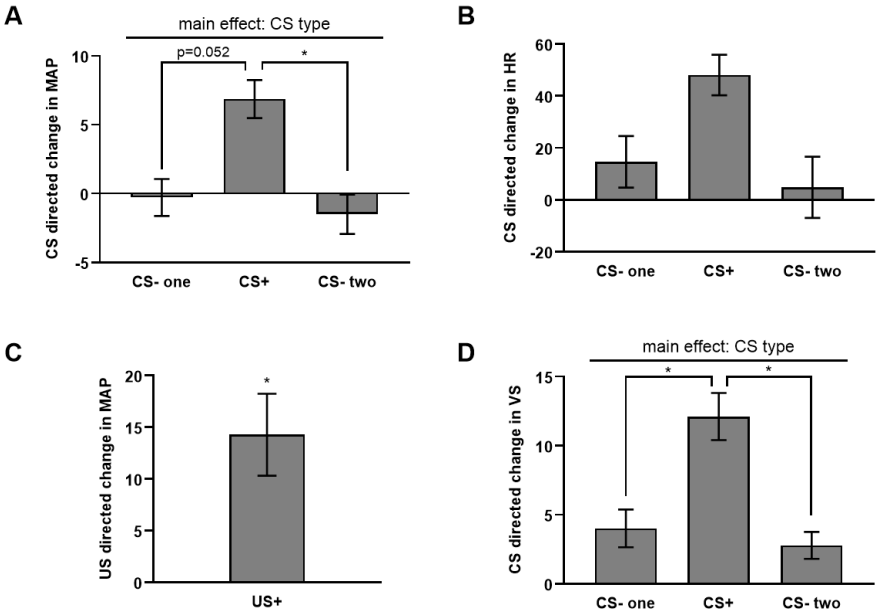


Figure 5-12 Animals successfully acquired conditioned aversive Fear Discrimination. Data from CS-/CS+/CS- sessions immediately prior to experimental manipulations. Relevant graphs show mean ± SEM. N=4. **A** Animals successfully discriminated between CS types based on CS directed MAP responses (effect of CS type: $F_{1.826,5.477}$=10.37, p=0.015). Sidak's multiple comparisons test revealed a significant difference between the CS+ and the second CS- (p=0.044) and a trend towards a difference between the CS+ and the first CS- (p=0.052). Compared to the mean CS- response, there was a significantly higher MAP response to the CS+ (two-tailed paired *t*-test, p=0.013; not shown). **B** Animals showed a trend towards significant discrimination based on HR values (effect of CS type: $F_{1.307,3.922}$=6.96, p=0.056). Compared to the mean CS- response, there was a significantly higher HR response to the CS+ (two-tailed paired *t*-test, p=0.010; not shown). Nevertheless, given the sensitivity of MAP responses to reflect conditioned discrimination (and the lack of effect of over-activation on MAP levels in the neutral condition; **Chapter 3**), MAP values were used as the principal cardiovascular measure in the Fear Discrimination study. **C** During the US+ period, animals exhibited cardiovascular arousal over-and-above levels shown during the CS+ (US directed change in MAP; one-sample *t*-test *vs.* 0, p=0.037). **D** Animals showed behavioural discrimination between CS types based on CS directed VS responses (effect of CS type: $F_{1.216,3.649}$=17.14, p=0.016). Sidak's multiple comparisons test revealed a significant discrimination between the CS+ and both the first (p=0.045) and second (p=0.047) CS-. Compared to the mean CS- response, there was a significantly higher VS response to the CS+ (two-tailed paired *t*-test, p=0.022; not shown).

5.4.8 SgACC/25 over-activation systematically increased cardiovascular and behavioural arousal during Fear Discrimination testing

Over-activation of sgACC/25 had no significant effect on CS directed MAP or VS responses compared to control infusions of saline vehicle (**FIGURE 5-13A, B**), nor was there any effect on the US directed response (mean ± SEM difference between control and over-activation: -3.2 ± 1.6mmHg, NS). However, for both MAP and VS measures, absolute values were typically higher during *both* the baseline and CS periods (baseline period MAP showed a trend towards a main effect of manipulation, p=0.052, whereas all other measures showed a significant main effect of manipulation; **FIGURE 5-13C, D**), indicating a contextual effect not specific to the CS period. To determine whether the effects on MAP arousal were present throughout the session, MAP values were plotted across the entire ~500s period. This plot (**FIGURE 5-13E**) indicated that MAP values were significantly, systematically higher following over-activation compared to control infusions.

5.4.9 SgACC/25 over-activation may impair recovery from a stressor

To determine whether the recovery from a stressor (US+) was affected by sgACC/25 over-activation, the 10s period following US termination was analysed. Specifically, a ratio was calculated comparing MAP value in the final 1s bin of the US+ period, to the MAP value in the subsequent ten, 1s bins following its termination. These ratios were calculated to try and account for the generalised effect over-activation had to increase MAP arousal. Analysis of this period indicated that, following US+ termination, MAP values remained higher for longer in the case of sgACC/25 over-activation compared to control infusions (a significant time x manipulation interaction, together with a longer time taken to reach pre-US+ levels of arousal; **FIGURE 5-13F**). This tentatively suggests that recovery from a stressor is slowed following sgACC/25 over-activation.

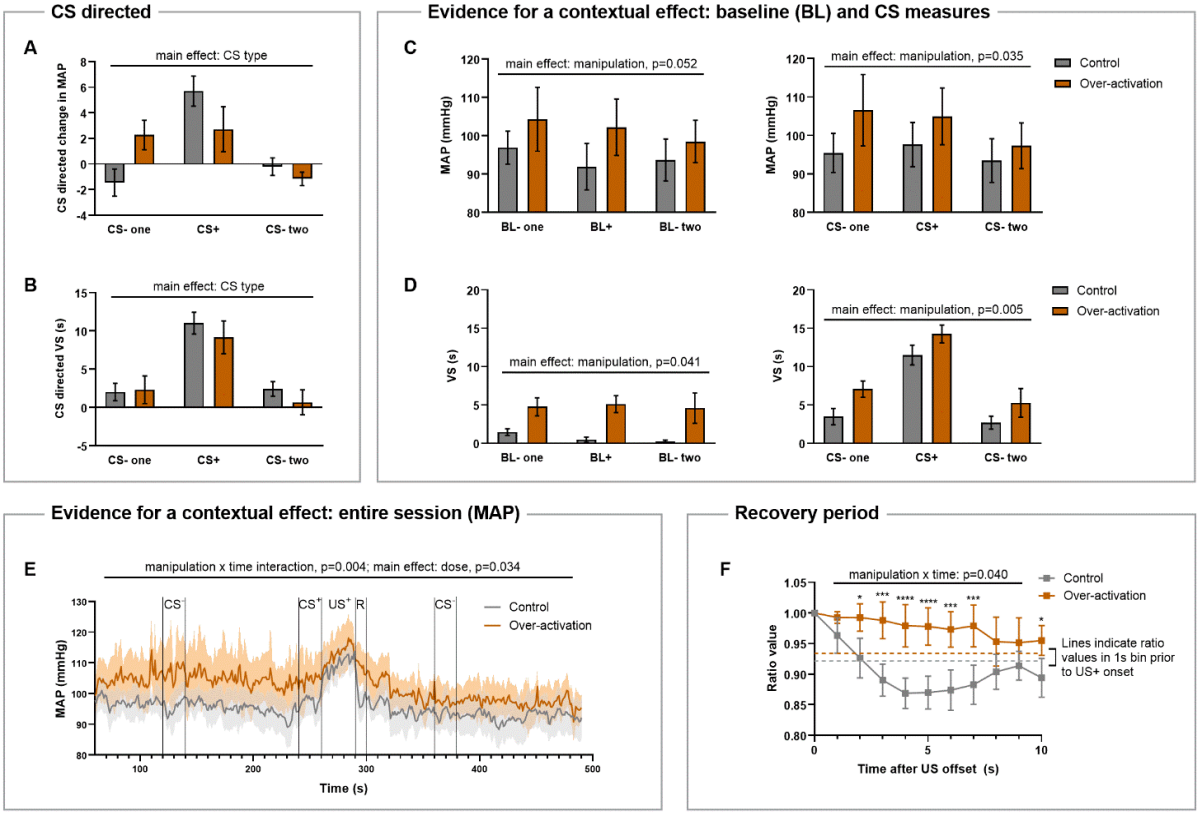


Figure 5-13 SgACC/25 over-activation enhances cardiovascular and behavioural arousal during an aversive Pavlovian Fear Discrimination paradigm. Relevant graphs show mean ± SEM. N=4. **A** SgACC/25 over-activation had no significant effect on CS directed MAP responses (manipulation × CS type, $F_{2,6}$=2.66, p=0.149; main effect of CS type maintained: $F_{2,6}$=19.28, p=0.002). **B** SgACC/25 over-activation also had no significant effect on CS directed VS responses (manipulation × CS type, F<1, NS; main effect of CS type maintained: $F_{2,6}$=16.58, p=0.004). **C** SgACC/25 over-activation tended to increase MAP during both baseline (left; manipulation × BL type, F<1, NS; main effect of manipulation: $F_{1,3}$=9.84, p=0.052) and CS (right; manipulation × BL type, $F_{2,6}$=1.12, p=0.386; main effect of manipulation: $F_{1,3}$=13.47, p=0.035) periods. **D** SgACC/25 over-activation significantly increased VS behaviour during both baseline (left; manipulation × BL type, F<1, NS; main effect of manipulation: $F_{1,3}$=11.81, p=0.041) and CS (right; manipulation × BL type, F<1, NS; main effect of manipulation: $F_{1,3}$=54.46, p=0.005) periods. **E** Given that sgACC/25 over-activation was associated with increased arousal during both baseline and CS periods, second-by-second MAP values were plotted across the entire session. MAP arousal was significantly elevated but the magnitude of this decreased across the session (manipulation × time, $F_{1,3814}$=8.33, p=0.004; main effect of dose: $F_{1,3.1}$=3.1, p=0.034). **F** Analysis of the 10s post-US+ recovery period indicated that the recovery of MAP arousal (calculated as a ratio to the MAP response in the final 1s bin of the US+) was significantly slower following sgACC/25 over-activation compared to control conditions (manipulation x time, $F_{9,27}$=2.38, p=0.040), with Sidak's multiple comparisons test revealing a significant difference in MAP arousal at 2s (p=0.028), 3s (p<0.001), 4s (p<0.0001), 5s (p<0.0001), 6s (p<0.001), 7s (p<0.001) and 10s (p=0.044) time-bins. Dashed lines indicate the ratio value of the final 1s time-bin prior to US+ onset compared to the final 1s of the

US+ period. Whilst the mean level of MAP arousal reached this value by the second/third time-bins in control conditions, the mean MAP arousal failed to reach this level across the entire 10s recovery period following sgACC/25 over-activation.

5.4.10 SgACC/25 over-activation profoundly increases anxiety responses to the HI

Over-activation of sgACC/25 enhanced responsivity of marmosets to the HI, as assessed by an increased anxiety score derived from EFA (**FIGURE 5-14A**). The increase in anxiety score was driven by the following measures:

- **Reduced TSAF** (**FIGURE 5-14B**). Mean ± SEM difference, over-activation – control: -24.4 ± 6.6%. Two tailed paired *t*-test, p=0.008.
- **Increased TSAB** (**FIGURE 5-14C**). Mean ± SEM difference, over-activation – control: 24.0 ± 8.5%. Two tailed paired *t*-test, p=0.009.
- **Increased average height** (**FIGURE 5-14D**). Mean ± SEM difference, over-activation – control: 15.5 ± 4.2cm. Two tailed paired *t*-test, p=0.008.

These measures indicate that animals were spending significantly more time high up and at the back of the cage. Also contributing to the enhanced anxiety score was a small but significant decrease in locomotion (**FIGURE 5-14E**) seen in seven out of eight animals (mean ± SEM difference, over-activation – control: -3.3 ± 1.3%; two-tailed paired *t*-test, p=0.040). Decreases in locomotion reflect increased 'stillness' and reduced approach of the marmoset towards the HI (Santangelo et al., 2016). Bobs (**FIGURE 5-14F**) and vocalisations (**FIGURE 5-14G-K**) showed more variation – none of these measures significantly differed between control and over-activation conditions.

The responses of animals across all conditions reported here are shown in **TABLE 5-2**.

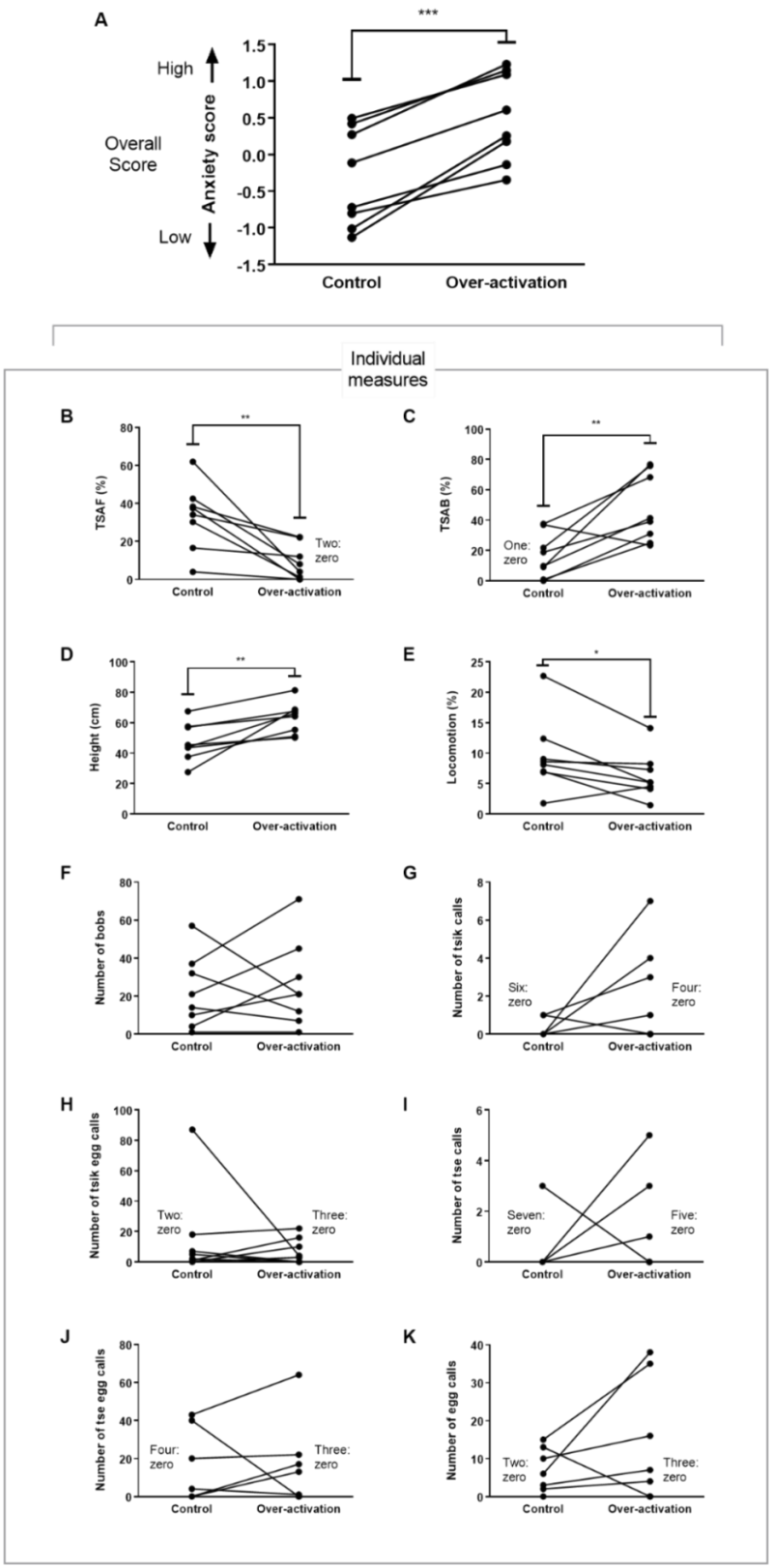


Figure 5-14 SgACC/25 over-activation increases anxiety responses to an HI.

Overall anxiety score shown top, with individual measures below. P values reported from two-tailed paired *t*-tests. N=8. **A** SgACC/25 over-activation increased anxiety responses to an HI, regardless of the level of anxiety animals exhibited under control conditions ($p<0.001$). **B** Time spent at front (TSAF, %; $p=0.008$). **C** Time spent at back (TSAB, %; $p=0.009$). **D** Height (cm; $p=0.008$). **E** Locomotion (%; $p=0.040$). **F** Number of bobs (count; $p=0.656$). **G** Number of tsik calls (count; $p=0.129$). **H** Number of tsik egg calls (count; $p=0.485$). **I** Number of tse calls (count; $p=0.402$). **J** Number of tse egg calls (count; $p=0.857$). **K** Number of egg calls (count; $p=0.231$)

Subject/manipulation	EFA	TSAF, %	TSAB, %	Height, cm	Locm., %	Bobs	Tsik	Tsik-egg	Tse	Tse-egg	Egg
Subject 1											
25 Saline	-1.01	61.94	9.82	45.35	8.6	4	0	0	0	0	0
25 DHK	0.26	4.00	41.38	50.17	8.23	30	1	10	3	13	0
Subject 2											
25 Saline	-1.13	42.49	0.00	27.52	1.76	1	0	0	0	0	0
25 DHK	0.18	8.02	31.02	68.73	4.51	1	4	3	0	0	0
Subject 3											
25 Saline	-0.72	38.29	0.86	43.64	9.03	10	0	1	0	4	2
25 DHK	-0.14	22.27	24.98	51.08	7.29	21	7	16	5	1	4
Subject 4											
25 Saline	-0.80	34.04	36.98	37.66	22.7	14	0	5	0	0	13
25 DHK	-0.35	22.08	23.40	55.38	14.11	7	0	0	0	0	0
Subject 5											
25 Saline	0.27	16.55	18.83	57.08	12.38	37	0	18	0	20	15
25 DHK	1.23	12.05	39.05	67.52	5.2	71	0	22	0	22	35
25 DHK + Ketamine	1.11	7.02	36.89	58.75	6.18	69	0	0	0	54	18
Subject 6											
25 Saline	-0.11	30.22	21.75	43.97	8.12	32	1	7	3	40	6
25 DHK	0.61	1.23	75.69	66.11	5.2	12	0	0	0	0	38
25 DHK + Ketamine	0.65	0.00	93.20	71.28	2.35	3	0	0	0	0	11
Subject 7											
25 Saline	0.49	37.51	9.11	67.53	7	57	0	87	0	0	3
25 DHK	1.09	0.00	76.71	81.36	1.44	21	0	4	0	17	7
25 DHK + Ketamine	1.87	0.00	82.81	80.35	2.32	75	0	41	0	40	1
Subject 8											
25 Saline	0.42	3.97	37.54	57.57	6.86	21	1	2	0	43	10
25 DHK	1.15	0.00	68.26	64.22	4.12	45	3	0	1	64	16
25 DHK + Ketamine	0.97	0.00	43.76	64.64	1.88	39	0	0	1	41	20

Table 5-2 HI behaviours of all eight subjects for all conditions. Subject numbers correspond to those outlined in **Chapter 2: General Methods** (cohort one, and Subject 16 from cohort two).

5.4.11 Ketamine does not reverse increases in anxiety associated with sgACC/25 over-activation

To assess the responsivity of a specific component of the sgACC/25 over-activation induced phenotype to treatment, four animals were injected with ketamine, and 1 day later were tested on the HI paradigm combined with sgACC/25 over-activation (**FIGURE 5-15A**). This timepoint corresponds to one of the timepoints at which ketamine successfully reversed anticipatory anhedonia (**Chapter 4**). Ketamine administration one day prior to over-activation had no effect on anxiety scores in the HI test – in all four animals, the anxiogenic effect associated with sgACC/25 over-activation was still present (**FIGURE 5-15B**).

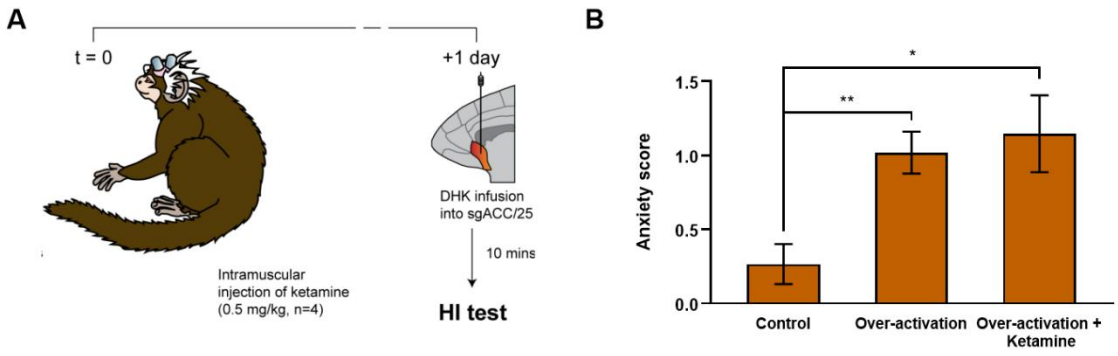


Figure 5-15 Ketamine does not reverse increases in anxiety associated with sgACC/25 over-activation. Relevant graphs show mean ± SEM. N=4. **A** Animals received a single intramuscular injection of ketamine, followed by over-activation of sgACC/25 and HI testing 1 day later. **B** Treatment type had a significant effect on anxiety scores ($F_{1.127,3.380}$=15.8, p=0.022). The result for over-activation alone is shown for this cohort of four animals (middle bar), demonstrating a significant effect to increase anxiety scores (p=0.006). Over-activation following ketamine administration 1 day earlier still resulted in significantly elevated anxiety scores (right bar, p=0.046). Additionally, there was no difference in anxiety scores between over-activation alone and over-activation following ketamine administration (p=0.939).

5.5 DISCUSSION

To summarise, the major findings of the studies presented in this chapter are as follows:

- SgACC/25 over-activation systematically enhances cardiovascular and behavioural arousal during the extinction of conditioned fear (Snake Extinction), the effects of which carry over to extinction recall;
- SgACC/25 over-activation systematically enhances cardiovascular and behavioural arousal during a novel aversive Pavlovian conditioning (Fear Discrimination) paradigm;
- SgACC/25 over-activation profoundly increases anxiety as measured by intolerance of uncertainty during exposure to an unfamiliar HI; and
- When tested on this specific aspect of the enhanced negative affect associated with sgACC/25 over-activation, ketamine fails to ameliorate associated impairments.

Together with these findings, there are two additional tentative conclusions implied by the results of these studies:

- Whilst having no effect on cortisol levels in neutral conditions, sgACC/25 over-activation appears to elevate cortisol levels in aversive contexts; and
- SgACC/25 over-activation may slow stress recovery following presentation of an aversive stimulus.

The data presented in this chapter therefore causally implicate sgACC/25 over-activity in the elevated negative affect considered a hallmark of anxiety and mood disorders.

5.5.1 SgACC/25 over-activity enhances cardiovascular and behavioural arousal to aversive contexts

We explored the consequences of sgACC/25 over-activity on (i) fear extinction during the Snake Extinction paradigm and (ii) fear learning/stress recovery during an aversive Pavlovian Fear Discrimination paradigm.

In the Snake Extinction paradigm, cardiovascular arousal was not CS specific as evidenced by the variability in CS directed MAP values during acquisition. Instead, MAP arousal responses were evidenced in the absolute MAP values following snake presentation. This suggests that the increase in cardiovascular arousal observed in CS periods during acquisition – together with its decline during extinction/extinction recall – likely reflects changing responses to the context. The lack of reliable CS specific cardiovascular conditioning may be related to (i) latent inhibition from three pre-acquisition exposures of the CS; (ii) the use of a single, short acquisition session to acquire the CS/US association (consisting of only 6 trials); or (iii) the highly aversive nature of the US, which may promote

fear generalisation from cue to context. Such factors, either acting alone or in combination, may make it difficult for animals to resolve cue-context competition during learning. Behaviourally, VS proved to be a better measure of CS specific fear – animals successfully acquired CS directed VS responses. Note that there also was some evidence of increased baseline VS following snake presentation which would reflect a response to the context (as it was exhibited outside of the CS period). The mixed pattern of cue- (VS) and context- (MAP and to some extent, VS) dependent learning is consistent with previous work using the Snake Extinction paradigm in the present author's laboratory (Wallis et al., 2017).

Given the characteristics of the learning animals exhibited on this paradigm, the enhanced cardiovascular/behavioural arousal during the CS period associated with sgACC/25 over-activation likely reflects an increase in both context- and cue-associated arousal. This is further corroborated by the systematic nature of elevated MAP and VS arousal responses across the session and in baseline periods. On subsequent extinction recall days, MAP/VS arousal was also elevated but the magnitude of the increase appeared to decay across session, suggesting that animals were continuing to extinguish on the following day. The context-associated effects of sgACC/25 over-activity are consistent with studies in humans which show elevated sgACC activity associated with contextual conditioning (Alvarez et al., 2008) and during sustained or unpredictable threat (i.e. threats not predicted by discrete cues) (Alvarez et al., 2011; Hasler et al., 2007b). In comparison, dACC has been implicated in cue-specific differential autonomic responses to CSs but not to contexts (Milad et al., 2007b). Note however that the subgenual region associated with contextual threat in these studies is not always sgACC/25 – in some cases, the zone of elevated activity is more rostral in sgACC/10.

In the Fear Discrimination paradigm, CS specific conditioning was evident in both CS directed MAP and VS responses, but this was seemingly unaffected by sgACC/25 over-activation. However, when the absolute MAP and VS values were assessed for baseline and CS periods separately, it was evident that there was a non-specific systematic increase in MAP and VS responses (in the cardiovascular domain, this was also apparent when MAP responses were plotted across the entire session). The effects of sgACC/25 over-activation on the Fear Discrimination paradigm also appear to be related to elevations in MAP/VS arousal directed to the context.

Therefore, across two different paradigms, it is apparent that sgACC/25 over-activation enhances cardiovascular and behavioural arousal when animals are in aversive contexts. Note that the nature of the elevated cardiovascular arousal seems to be different in aversive contexts compared to the neutral condition, since elevated HR – but not MAP – responses were observed in the neutral condition (**Chapter 3**). The differential effects of sgACC/25

over-activation on MAP and HR across neutral and aversive-associated contexts may relate to the differential contributions of the parasympathetic and sympathetic branches of the autonomic nervous system to the regulation of HR compared to the regulation of total peripheral resistance (and therefore MAP) in the vasculature. Parasympathetic fibres innervate the heart and a relatively small number of vessels, so the influence of the parasympathetic division is restricted to chronotropic influences on HR. Neural control of BP, by contrast, is largely exerted via the sympathetic nervous system because sympathetic fibres diffusely innervate arterioles, meaning that alterations in total peripheral resistance can be exerted by changing activity in the sympathetic branch of the autonomic nervous system (Thomas, 2011). It is possible that in neutral conditions, sgACC/25 has a predominant effect on parasympathetic tone to modulate HR, whereas in aversive contexts (where there is expected to be an increased sympathetic drive) sgACC/25 activity alters the gain within the sympathetic system to modulate MAP.

Broadly speaking, these results are the opposite to those expected from data pertaining to activity within the putative rodent homologue – IL – during extinction. Electrophysiological and immunohistochemical evidence suggests that increased activity within IL correlates with reduced fear during extinction retrieval (Holmes et al., 2012; Knapska et al., 2012; Milad and Quirk, 2002). Pharmacological (Thompson et al., 2010) and optogenetic (Do-Monte et al., 2015) over-activation of IL also enhances extinction learning. Rodent studies, therefore, suggest that *increased* IL activity is associated with *reduced* fear responding. The results reported herein suggest the opposite is true for the anatomical primate homologue – *increased* activity in sgACC/25 *enhances* fear responding – and therefore calls into the question the idea that sgACC/25 and IL are functionally analogous.

It is important to emphasise that the results are also different at a subtle level: whilst IL manipulations alter rates of extinction learning, no such effect was observed following over-activation of sgACC/25. The different effects observed may be due factors including:

- **Functional differences between primate sgACC/25 and rodent IL**. Beyond differences in the direction of the effect, a difference in the ‘nature’ of the effect could also be explained by functional differences between sgACC/25 and IL.
- **Differences in conditioning procedures and the associations that are formed.** As discussed above, cardiovascular and behavioural readouts indicate that during acquisition sessions of the Snake Extinction test, both contextual and cue-specific associations are formed. If rodent studies obtain a more CS-specific (or, indeed, more context-specific) pattern of learning, this may result in different effects.
  At this point, it is worth mentioning that it is not clear from classic rodent fear conditioning studies whether the associations that are formed and tested are CS

specific. Typically, researchers use different training and testing chambers to make the contexts different and therefore the expression of fear at test predominantly CS directed – however, baseline levels of freezing are rarely reduced to zero, and the problem is made worse when strains of rats/mice are used which show a high degree of fear generalisation. A study by Michael Fanselow and colleagues has shown that in mice there is a clear positive interaction between baseline (context-directed) fear and CS (tone) induced fear, such that baseline levels of freezing seriously confound measures of CS-specific fear (Jacobs et al., 2010).

The contextual effects of sgACC/25 over-activation have relevance to clinical disorders associated with elevated anxiety. Both discrete and contextual Pavlovian processes are thought to contribute to anxiety disorders (Vervliet et al., 2013) – but contextual arousal is particularly relevant to psychopathologies characterized by sustained or 'free-floating' anxiety when there is no clear threat-eliciting stimulus; exemplified by GAD or symptom clusters of PTSD that involve excessive arousal in the absence of threat-eliciting cues (Kroes et al., 2017). The generalised elevation in arousal associated with sgACC/25 over-activation may be consistent with the exaggerated responses to ineffable threat seen in these disorders.

5.5.2 SgACC/25 over-activity may impair stress recovery

The reliable increases in US+ directed arousal in the Fear Discrimination paradigm provided us with an opportunity to investigate cardiovascular recovery following aversive stimulus presentation. Whilst the generalised effect of sgACC/25 over-activation to elevate MAP complicates interpretation of the stress recovery period (as the cardiovascular response potentially has a smaller range in which to decline), analysis of the 10s post-US period suggests that the cardiovascular arousal associated with US+ presentation takes longer to decay following sgACC/25 over-activation. This tentatively implicates elevated sgACC/25 activity in impaired stress recovery – a phenotype which is reliably identified in depressed patients (Burke et al., 2005). Further caution is also warranted, as it is possible that this effect is not specific to the decay of arousal following *aversive* USs – to investigate this, further work is required to determine if sgACC/25 over-activation affects cardiovascular arousal following an *appetitive* US.

5.5.3 SgACC/25 over-activity increases anxiety as measured by intolerance of a HI

In response to an HI, marmosets exhibit a complex repertoire of behaviours. An EFA was used to predict the extent to which marmosets' responses are driven by underlying latent factors. One factor was extracted, and the pattern in which individual behaviours load onto this factor suggest that it represents the animal's anxiety response. The face validity of this EFA-extracted measure is evidenced by, for example, the strong negative loading of TSAF

and the strong positive loading of TSAB. These measures are sensitive to classic anxiolytic agents (Carey et al., 1992), also attesting to the EFA score's predictive validity.

To assess the role of sgACC/25 over-activity in anxiety behaviours associated with intolerance of uncertainty, the behavioural profile of animals during confrontation with an HI was compared during control and over-activation conditions. Based on the anxiety score extracted from EFA, over-activation of sgACC/25 profoundly enhanced anxiety responses. The most consistent effects observed were on distance measures including TSAF, TSAB and height. There was also a small but significant reduction in locomotion, reflecting increased 'stillness.' These data are consistent with work in macaques, demonstrating that sgACC/25 activity is related to stress responses measured during HI exposure (Jahn et al., 2010).

The enhanced anxiety responses associated with this temporary manipulation are not necessarily maladaptive: for example, upward flight (increased height) is a normal defensive response to predators (Searcy and Caine, 2003). Indeed, enhanced activity in sgACC/25 is associated with 'normal' negative affect as well as 'abnormal' negative affect in psychiatric disorders (Mayberg, 1997) – so whether this acute manipulation represents a sufficient change to be considered 'abnormal' is not clear. Clinically, the definition of an 'abnormal' (maladaptive) behaviour relates to an impairment in everyday function (American Psychiatric Association, 2013). However, the cognitive and neural factors which tip normal adaptive responses into maladaptive ones are undoubtedly multi-factorial, complex and dependent on changes within a distributed network of neural structures rather than one single brain region. Nevertheless, these data do causally implicate sgACC/25 over-activity in the enhanced intolerance of uncertainty which *could* be maladaptive in the context of mood/anxiety disorders.

5.5.4 Ketamine fails to reverse over-activation associated enhanced anxiety responses to an HI

Despite successfully reversing the anticipatory anhedonic impairments as described in **Chapter 4**, a single dose of ketamine failed to ameliorate the increased anxiety induced by sgACC/25 over-activation in response to an HI. As discussed above, evidence for ketamine as an efficacious treatment for anxiety disorders remains in early stages, although a small number of studies have shown promising initial results (Glue et al., 2017, 2018). These studies have assessed the beneficial effects of ketamine using questionnaires (such as the Hamilton Anxiety Rating Scale, HARS) (Hamilton, 1959) and interview-based reports. Whilst the construct validity of questionnaires is always a concern, the HARS has shown convergent validity with clinician-rated and self-report measures of anxiety symptoms (Shear et al., 2001). Given that intolerance of uncertainty shows strong correlation with HARS

scores (Boswell et al., 2013), the lack of efficacy of ketamine observed here is less likely to be related to a fundamental difference in construct being assessed although this is always possible. The difference in efficacy is also unlikely related to the time-course of dosing– Glue and colleagues report an efficacy within one hour of ketamine administration, with sustained improvements over one week (Glue et al., 2018). According to these data, one would expect a sustained effect of a single dose of ketamine 1 day later (and indeed, we obtained this in **Chapter 4**). Future work could assess the efficacy of ketamine at earlier timepoints, to see if there is a more transient effect to ameliorate elevated anxiety in this HI preparation.

These results have implications for the mechanistic basis of anhedonia and anxiety induced by sgACC/25 over-activation. Given that ketamine successfully reverses anticipatory anhedonia but fails to reverse the enhanced anxiety at the 1-day timepoint, this implies that there are fundamentally different mechanisms at play underlying the two effects (**FIGURE 5-16**). More explicitly, if a common mechanism was involved in the induction of both changes, ketamine would be expected to reverse *both*. Therefore, two separate mechanisms must be responsible for the anhedonic/anxiogenic changes. Future work using ^{18}F-FDG imaging to investigate the downstream neural consequences of sgACC/25 over-activation both with and without ketamine in aversive settings is warranted, to compare the pattern of metabolic change with those observed in appetitive settings (**Chapter 4**).

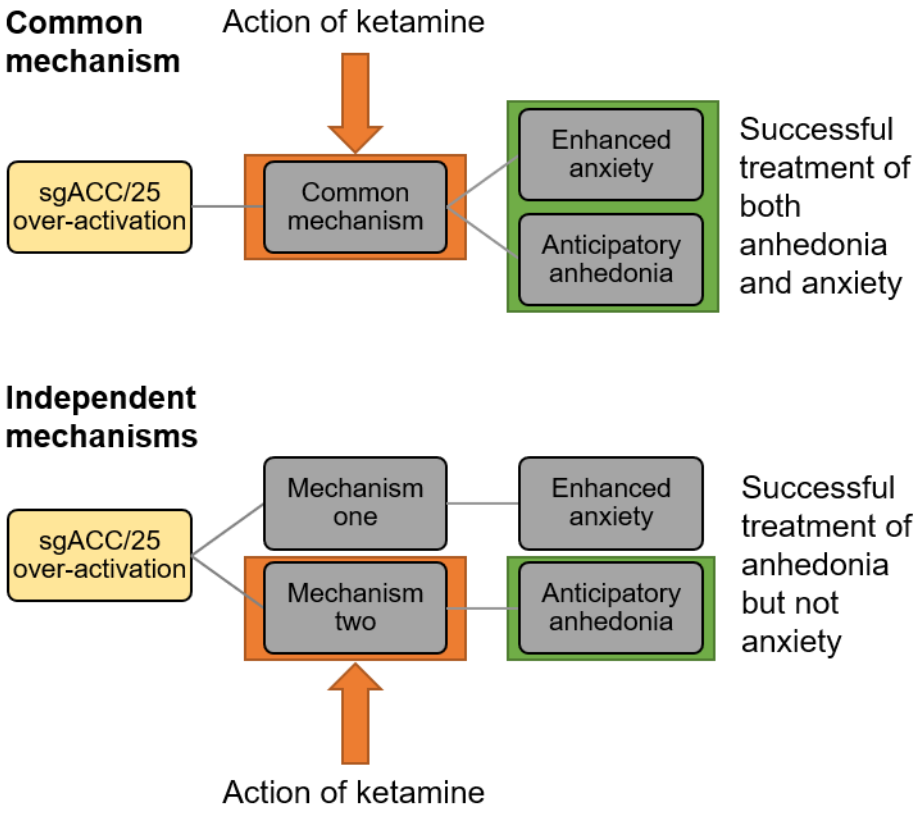


Figure 5-16 Mechanisms of action of ketamine in the context of sgACC/25 over-activation. If sgACC/25 over-activation was causing enhanced anxiety and anticipatory anhedonia by a common mechanism (top), ketamine would be expected to ameliorate both impairments. By contrast, if two independent pathways were involved in enhanced anxiety and anticipatory anhedonia, it is feasible that ketamine could act to ameliorate one symptom cluster through effects restricted to one mechanism whilst failing to ameliorate the other symptom cluster (bottom). Given that ketamine successfully ameliorated anticipatory anhedonic symptoms (**Chapter 4**) but failed to ameliorate enhanced anxiety (present chapter), the data presented in this thesis support the latter suggestion.

5.5.5 SgACC/25 over-activity may potentiate HPA axis activity in aversive contexts

As discussed in **Chapter 3**, sgACC/25 over-activation had no effect on salivary cortisol levels in the neutral condition. However, following extinction sessions on the Snake Extinction paradigm, salivary cortisol levels were elevated compared to pre-extinction levels. This suggests that sgACC/25 modulates activity within the HPA axis in aversive contexts. This is consistent with work in rodents, implicating regions of the mPFC (including IL) in the regulation of cortisol levels during restraint stress but not in baseline conditions (Diorio et al., 1993).

The elevation in salivary cortisol associated with over-activation of sgACC/25 could be interpreted in two ways (**FIGURE 5-17**):

- **Model 1**: sgACC/25 provides a direct stimulatory input to CRH+ neurons of the hypothalamic PVN and is regulated by a negative feedback loop – the stimulatory input is exaggerated by sgACC/25 over-activation; or
- **Model 2**: sgACC/25 contributes to a positive feedback loop associated with acute stress. SgACC/25 provides inhibitory input to an intermediate structure which inhibits the HPA axis. When rapid rises of cortisol levels are needed in situations of acute stress, sgACC/25 is activated, enhancing inhibitory input to this intermediate structure thereby disinhibiting the HPA axis. In model 2, cortisol would not negatively feedback onto sgACC/25.

Anatomical data supports a direct input from vmPFC subregions including sgACC/25 to the hypothalamus, lending support to Model 1. Functional data from McKlveen and colleagues also lends support to Model 1, as inhibitory feedback of cortisol (via GCRs) onto the putative sgACC/25 homologue IL constitutes a negative feedback loop during situations of stress (McKlveen et al., 2013), although this data is confounded by apparent differences in function between IL and sgACC/25.

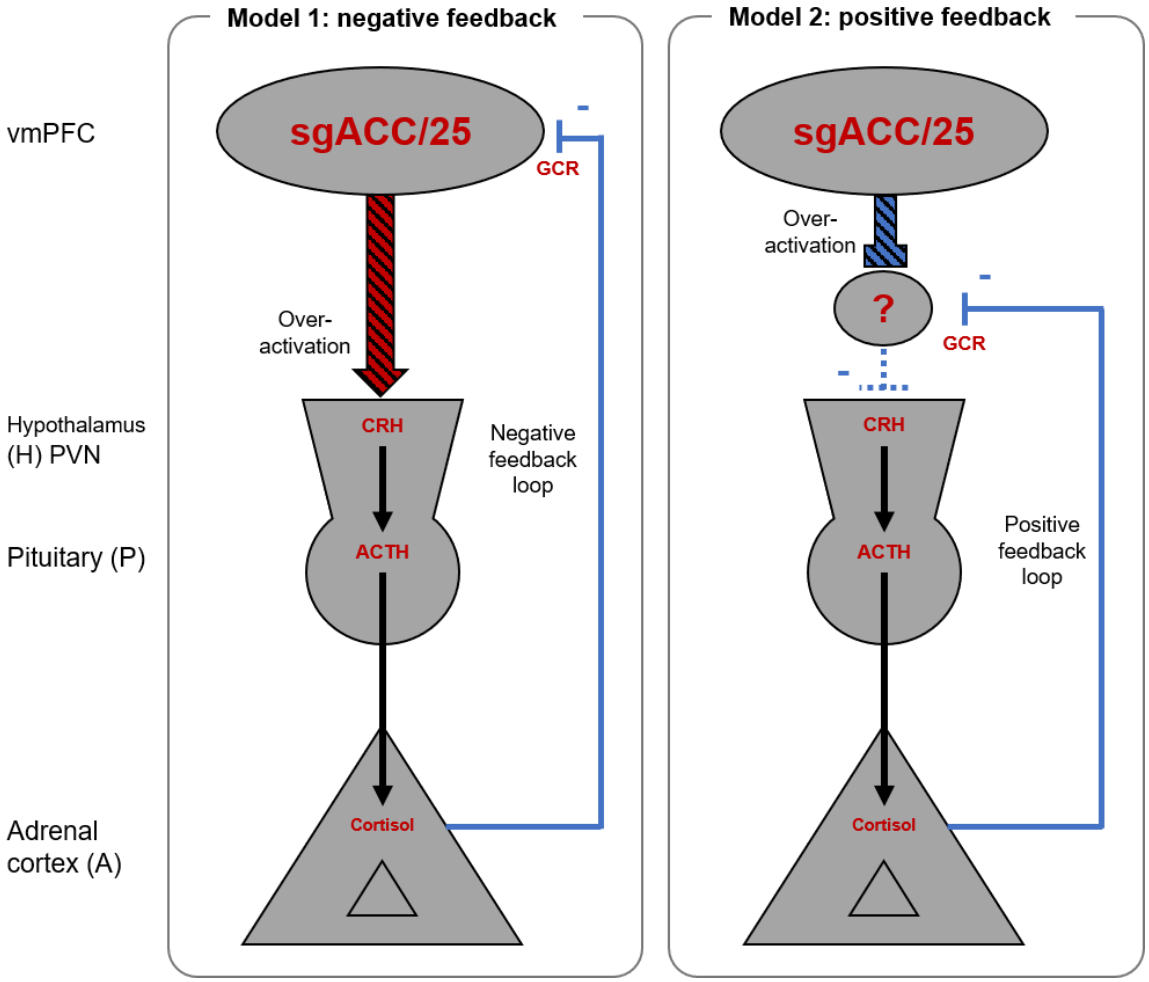


Figure 5-17 The relationship between sgACC/25, the HPA axis and peripheral cortisol levels. In **Model 1**, sgACC/25 provides direct excitatory input to the HPA axis and is a target for the negative feedback effects of circulating cortisol. Over-activation of sgACC/25 elevates excitatory output to the PVN of the hypothalamus, which in turn stimulates more ACTH and cortisol release. Under normal circumstances, this elevated cortisol would negatively feedback onto sgACC/25 (as well as other structures) to maintain homeostatic ranges of cortisol concentrations, mediated by GCRs. In **Model 2**, sgACC/25 contributes to a positive feedback loop facilitating rapid elevations in cortisol levels associated with acute stress. In this model, an intermediate structure (such as the hippocampus) normally provides tonic inhibition of the HPA axis – this structure is inhibited by feedback from peripheral cortisol, together with inhibitory input from sgACC/25. When sgACC/25 is over-activated, the inhibitory input to this intermediate structure is enhanced, thereby disinhibiting CRH+ neurons in the PVN and potentiating HPA axis output.

5.6 CONCLUSION

The data presented in this chapter causally implicate sgACC/25 in enhanced arousal in aversive contexts associated with sustained threat – namely, enhanced cardiovascular and behavioural arousal in contexts associated with aversive stimuli (Snake Extinction/Fear Discrimination) – together with enhanced arousal associated with intolerance of uncertainty during anxiety-provoking situations (HI). These data also implicate sgACC/25 over-activity in prolonged stress recovery following presentation of acute stressors, and in elevated HPA axis activity associated with an exaggerated stress response. Further work is warranted to determine the precise contribution sgACC/25 makes to contextual information processing, and to further elucidate the anatomical connectivity of sgACC/25 to structures involved in cardiovascular control and the regulation of the HPA axis.

6 BLUNTED REWARD AROUSAL AND ENHANCED ANXIETY FOLLOWING PERIPHERAL INJECTIONS OF CORTISOL

Abbreviation	Meaning
^{18}F-FDG PET	^{18}Fluorine-fluorodeoxyglucose positron emission tomography
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
Cort(Num)	Cortisol (dose in mg/kg)
CRH	Corticotropin releasing hormone
CS	Conditioned stimulus
DA	Dopamine
dACC	Dorsal anterior cingulate cortex
EFA	Exploratory factor analysis
GCR	Glucocorticoid receptor
HI	Human intruder
HPA	Hypothalamo-pituitary-adrenal
HR	Heart rate
IGT	Iowa Gambling Task
IL	Infralimbic (cortex)
MAP	Mean arterial pressure
MCR	Mineralocorticoid receptor
MID	Monetary incentive delay
NHP	Non-human primate
NS	Not significant
OFC	Orbitofrontal cortex
PFC	Prefrontal cortex
PL	Prelimbic (cortex)
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus (of the hypothalamus)
SEM	Standard error of the mean
sgACC	Subgenual anterior cingulate cortex
TSAB	Time spent at back
TSAF	Time spent at front
US	Unconditioned stimulus
vmPFC	Ventromedial prefrontal cortex

6.1 Abstract

The thesis so far has explored the consequences of sgACC/25 over-activity on anxiety and fear regulation, together with its effects on the anticipatory, motivational and consummatory elements of reward processing. However, it remains unclear (i) under what physiological circumstances is sgACC/25 activated and (ii) the precise mechanisms by which sgACC/25 over-activity result in anxiogenic and anhedonic changes. Given the relationship between vmPFC subregions and HPA axis regulation, we conducted experiments to determine whether peripheral injections of the glucocorticoid cortisol could induce anxiogenic and anhedonic changes akin to those observed following sgACC/25 over-activation. Should the effects of peripheral cortisol injection be similar, this would support a role for the HPA axis in the effects associated with sgACC/25 over-activation – either as a cause, or a consequence. We found that subcutaneous injections of 20mg/kg cortisol successfully elevated peripheral cortisol to peak circadian levels and to the physiological response to stress, as measured by salivary cortisol sampling. In an appetitive setting, acute elevations in cortisol induced the behavioural signs of anticipatory anhedonia but did not affect cardiovascular anticipatory arousal. Consummatory arousal remained intact as measured during appetitive Pavlovian conditioning and during the sucrose preference test. In an aversive setting, cortisol injections moderately increased anxiety towards an HI. Therefore, elevated peripheral cortisol levels are associated with broadly similar changes across aversive and appetitive settings to those observed during sgACC/25 over-activation, although there are notable differences. Altered HPA axis functioning may therefore be responsible for some – but not all – of aspects of the anxiety/anhedonia induced by sgACC/25 over-activation. These experiments provide a foundation upon which future work can build, to precisely delineate the physiological and pathophysiological interactions between the HPA axis and sgACC/25.

6.2 Introduction

Stress and dysregulation within the HPA axis has long been implicated in the aetiology and pathophysiology of depression and anxiety (Faravelli et al., 2012; Juruena, 2014; Keller et al., 2017; Varghese and Brown, 2001). The nature of this relationship remains unclear – are these stress-related disorders *caused* by dysregulation within the HPA axis, or do these disorders *cause* the dysregulation (**Figure 6-1**)? Either way, impaired functioning of negative feedback systems involved in regulating the activity within the HPA axis is becoming increasingly recognised as a critical feature of the physiological dysfunction associated with psychiatric disorders, as a cause or consequence. The failure of negative feedback leads to sustained high levels of stress hormones – particularly the glucocorticoid cortisol – which have deleterious consequences, including maladaptive physiological changes and negative mood (Keller et al., 2017).

In parallel to a growing understanding of the link between HPA axis activity and psychiatric disorders, there is an evolving appreciation for the importance of limbic structures in regulating HPA axis activity, including the vmPFC. In rodents, excitotoxic lesions of both PL and IL sectors of the vmPFC alter activity within the hypothalamic PVN neurons responsible for stimulating ACTH release from the anterior pituitary (Jankord and Herman, 2008). Both sectors have also been implicated in negative feedback control – glucocorticoids act via PL to regulate negative feedback during situations of acute stress only, whereas in IL, glucocorticoids exert negative feedback effects during both acute and chronic stress (McKlveen et al., 2013).

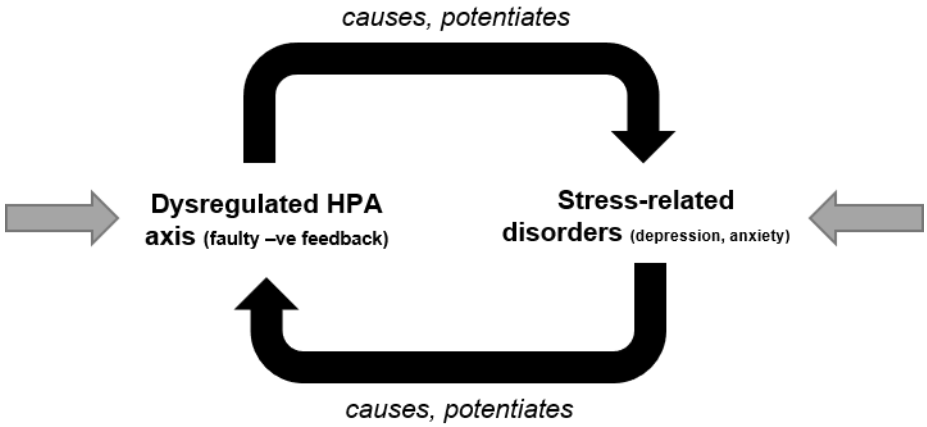


Figure 6-1 The relationship between dysregulation within the HPA axis and stress-related disorders such as depression and anxiety. Stress-related disorders psychiatric disorders may *cause* dysregulation within the HPA axis, or alternatively, dysregulation within the HPA axis may *cause* stress-related psychiatric disorders. Patients with these disorders could enter a maladaptive 'loop,' which may be difficult to escape: psychiatric disorders could potentiate dysregulation within the HPA axis and *vice-versa*. Note that a dysregulated HPA axis is not the only cause of these psychiatric disorders, just as these disorders themselves are not the only cause of a dysregulated HPA axis, and the grey arrows schematically represent the alternative routes through which patients could enter this loop.

The precise nature of the interplay between the vmPFC and the HPA axis, together with the role that dysfunction within these systems plays in stress-related disorders such as depression/anxiety, remains unclear. Evidence for a link between the two is further substantiated by several streams of data (from rodents, NHPs and humans) showing that the behavioural and cognitive functions mediated by sectors of the vmPFC are also deleteriously affected by stress. For instance:

- **Working memory**. In rats, chronic stress impairs spatial working memory through a D1 receptor mediated hypo-dopaminergic mechanism in the vmPFC (Mizoguchi et al., 2000).

- **Behavioural flexibility**. Chronic stress impairs reversal learning and set-shifting (Bondi et al., 2008; Danet et al., 2010), and normal performance of both of these behaviours is dependent on an intact vmPFC/OFC (Birrell and Brown, 2000; Floresco et al., 1997).
- **Decision making**. Administration of cortisol either systemically or directly into IL impairs performance on a rodent version of the IGT (Koot et al., 2014).
- **Habit formation**. Chronic stress promotes habit formation, accompanied by widespread atrophy of neurons in the IL and PL (Dias-Ferreira et al., 2009).
- **Emotional behaviours**. Chronic stress increases immobility time in the forced swim test (de Kloet and Molendijk, 2016), and GCR knockdown in IL has also been shown to increase immobility time (McKlveen et al., 2013).

Consider, therefore, the following lines of evidence. It is apparent that (i) dysregulated HPA axis activity is associated with increased susceptibility towards developing mood/anxiety disorders; (ii) dysfunction within the vmPFC is associated with mood/anxiety disorders; and (iii) the vmPFC is involved in the regulation of HPA axis activity. This begs the question as to whether vmPFC dysfunction is causal, resulting in depression- and anxiety-like changes through disruptions in HPA axis regulation; whether HPA axis dysfunction is causal, causing changes in vmPFC function which then result in depression/anxiety; or alternatively, whether the two are independent of one another and contribute to the deleterious phenotype entirely separately (**FIGURE 6-2**).

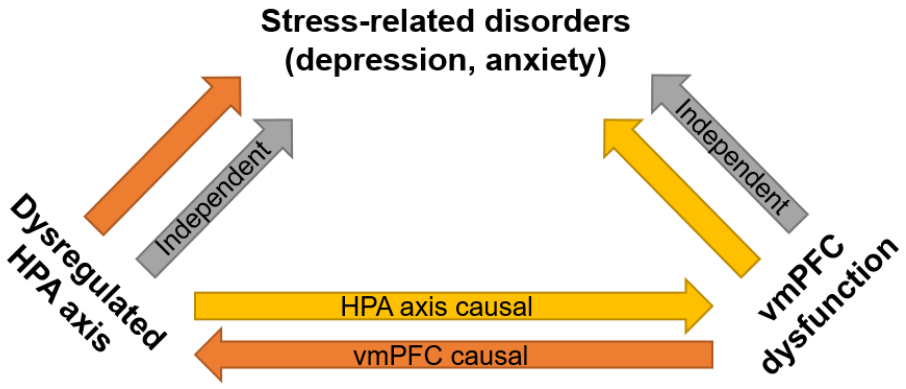


Figure 6-2 Causality: HPA axis and vmPFC dysfunction associated with depression and anxiety. An additional layer of complexity is introduced when the role of vmPFC dysfunction is considered in the context of HPA axis dysregulation and psychiatric disorders. Represented in orange is the pathway of causation if dysfunctional activity within the vmPFC is causing dysregulation within the HPA axis, in turn causing anxiety and depression; in yellow, the pathway of causation if dysregulation within the HPA axis is causing vmPFC dysfunction, in turn causing

anxiety and depression; and in grey, if the two changes are independently contributing to anxiety and depression.

From **Chapter 3** and **Chapter 5**, data in this thesis has provided preliminary evidence for a link between vmPFC over-activity and HPA axis activity. Whilst over-activity in sgACC/25 has no effect on salivary cortisol levels in an emotionally neutral condition, it does elevate cortisol levels following exposure to an aversive context (during Snake Extinction paradigm extinction sessions). These data reflect the first demonstration that over-activity in NHP vmPFC is causally linked to HPA axis output in contexts associated with negative affective valence.

The work in this chapter serves as a foundation for further work investigating the interaction between the vmPFC and HPA axis in regulating cortisol dynamics. The experiments presented here concern the consequences of acute elevations in peripheral cortisol levels on anxiety- and anhedonic-like behaviours in the marmoset monkey. Given the effects we have observed following over-activation of sgACC/25 to increase intolerance of uncertainty and anxiety (measured on the HI test) and reduce reward arousal (measured on the appetitive Pavlovian discrimination paradigm), we sought to determine whether peripheral injections of cortisol could mimic some – or all – of these changes. The experiments described herein are not designed to address the direction of causality related to HPA axis dysfunction, vmPFC dysfunction and anxiety/anhedonia symptoms: rather, they address whether the phenotype observed following acute elevations in peripheral cortisol (mimicking the endocrine response to an acute stressor) is in any way like the phenotype observed following sgACC/25 over-activation. In so doing, we sought to determine whether elevations in peripheral cortisol could explain some of the changes induced by this manipulation. These data are especially important in interpreting future data concerning the effects of vmPFC manipulations on the HPA axis.

6.3 METHODS

6.3.1 Subjects

Four marmosets (two male, two female) took part in this study. These marmosets were Subjects 9, 17, 18 and 19 of cohort three, described in **2.1.1 SUBJECTS**. The marmosets were housed and cared for as described in **2.1.2 HOUSING**.

6.3.2 Surgical procedures

Four marmosets underwent one surgical procedure as part of this study, to implant a telemetric blood pressure probe into the abdominal aorta. Subject 9 had previously undergone intracerebral cannulation surgery, but the implant detached post-surgery, and so Subject 9 was transferred to this cohort. All animals used in this study had also undergone stereotaxic surgery to infuse a DREADDs viral construct into sgACC/25 (see **TABLE 2-3**). The results of the DREADDs experiments are not reported in this thesis. However, if any DREADDs manipulations had taken place prior to cortisol manipulations, all behavioural and cardiovascular parameters had returned to normal before any manipulations pertinent to this chapter took place.

6.3.3 Behavioural testing apparatus and paradigms

Animals taking part in this study underwent (i) the appetitive Pavlovian discrimination task to assess anticipatory and consummatory arousal; (ii) the sucrose preference task to assess consumption on a task analogous to that used in rodents; and (iii) the HI test to assess anxiety responses. The appetitive Pavlovian discrimination and sucrose preference paradigms are described in **4.3.3 BEHAVIOURAL TESTING APPARATUS AND PARADIGMS**. The HI paradigm is described in **5.3.3 BEHAVIOURAL TESTING APPARATUS AND PARADIGMS**.

6.3.4 Drug treatments

Peripheral drug treatments were carried out as described in **2.4 DRUG TREATMENTS**. The pharmacological compounds used in experimental manipulations in this study were: 0.9% saline (vehicle control) and hydrocortisone hemisuccinate (cortisol; GCR agonist).

A 20mg/kg dose of cortisol was chosen as a common dose for all behavioural testing paradigms to achieve circulating cortisol levels in between the peak levels reached during normal circadian rhythms and in the response to stress observed in marmosets (Ash et al., 2018). This was based on work reported in another marmoset study, which used a 40mg/kg dose and induced supra-physiological cortisol levels (Saltzman and Abbott, 2009). In the appetitive Pavlovian conditioning paradigm, multiple doses were tested – 5mg/kg, 20mg/kg and 40mg/kg.

For all manipulations conducted in the context of behavioural testing paradigms, testing commenced one hour after cortisol injection. The time course of one hour is consistent with

mixed genomic and non-genomic effects of cortisol (Falkenstein et al., 2000). All injections were done in the mornings close together in time: between 08:30am and 10:00am for the appetitive Pavlovian discrimination task and the sucrose preference task, and between 11:00am and 12:00pm for the HI test. This was to minimise changes in baseline levels of cortisol (which fluctuate throughout the day) (Cross and Rogers, 2004).

6.3.5 Salivary cortisol sampling

In the manipulation check, salivary cortisol samples were taken and processed as described in **2.5 SALIVARY CORTISOL SAMPLING**. Specifically, a 'pre' salivary sample of cortisol was obtained immediately after injection of 20mg/kg cortisol. A 'post'-manipulation sample was taken one hour later.

6.3.6 Data acquisition and preliminary analysis

Telemetric data and behavioural data for the appetitive Pavlovian conditioning paradigm, together with behavioural data for the sucrose preference test, were collected and analysed as described in **4.3.6 DATA ACQUISITION AND PRELIMINARY ANALYSIS**. HI data were collected and analysed as described in **5.3.6 DATA ACQUISITION AND PRELIMINARY ANALYSIS**.

6.3.7 Statistical analysis

Salivary cortisol data collected for the manipulation check were analysed in two ways. Firstly, a two-way repeated measures ANOVA was conducted of the form $M_2 \times P_2$ where *M* is a factor with two levels (manipulation – saline or 20mg/kg cortisol) and *P* is a factor with two levels ('pre' or 'post'). Secondly, 'post':'pre' ratios were also calculated for saline control and cortisol manipulations. These were compared using a two-tailed paired *t*-test.

Statistical tests were conducted on appetitive Pavlovian conditioning data as described in **4.3.7 STATISTICAL ANALYSIS** except:

- Two-way repeated measures ANOVAs were the form $M_4 \times C_2$ (rather than $M_2 \times C_2$) as there were four levels of manipulation type (control, 5mg/kg, 20mg/kg and 40mg/kg cortisol) rather than two; and
- US data were compared using a one-way repeated measures ANOVA (rather than a two-tailed paired *t*-test) as there were four levels of manipulation type (control, 5mg/kg, 20mg/kg and 40mg/kg cortisol) rather than two.

Statistical tests were conducted on sucrose preference data as described in **4.3.7 STATISTICAL ANALYSIS**. Statistical tests were conducted on HI data as described in **5.3.7 STATISTICAL ANALYSIS**.

6.4 RESULTS

6.4.1 Subcutaneous cortisol injections successfully raised peripheral cortisol levels as measured by increases in salivary cortisol concentrations

Marmosets received a subcutaneous injection of saline vehicle control or 20mg/kg cortisol and the 'pre' sample of saliva was taken immediately after injection. The 'post' sample was taken after a wait time of one hour (**FIGURE 6-3A**). Injections of 20mg/kg cortisol successfully elevated salivary cortisol levels, as measured by a significant increase in cortisol concentration from the 'pre' to 'post' measurement, and compared to the 'post' measurement under control conditions (**FIGURE 6-3B**). This increase was also reflected in a significant increase in the 'post':'pre' ratio for cortisol injections *vs.* control (**FIGURE 6-3C**).

The mean ± SEM level of salivary cortisol achieved at the 'post' measurement following 20mg/kg cortisol injection was 342 ± 33µg/dL, equivalent to 9430 ± 916 nmol/L. In a recent study by Ash and colleagues, peak AM cortisol levels in marmosets were 7710 ± 6740 nmol/L (Ash et al., 2018). This means that the levels of cortisol achieved following 20mg/kg injections were equivalent to the highest physiological levels obtained during peak concentrations in the morning. Ash and colleagues also measured salivary cortisol levels following a mild stressor (being handled for weighing), but observed a cortisol *decrease* to levels of 2800 ± 700 nmol/L (the decrease presumably due to negative feedback mechanisms, or differences in physiological responses depending on the nature of the stressor). The cortisol levels we obtained were higher than this, although they are within the same order of magnitude.

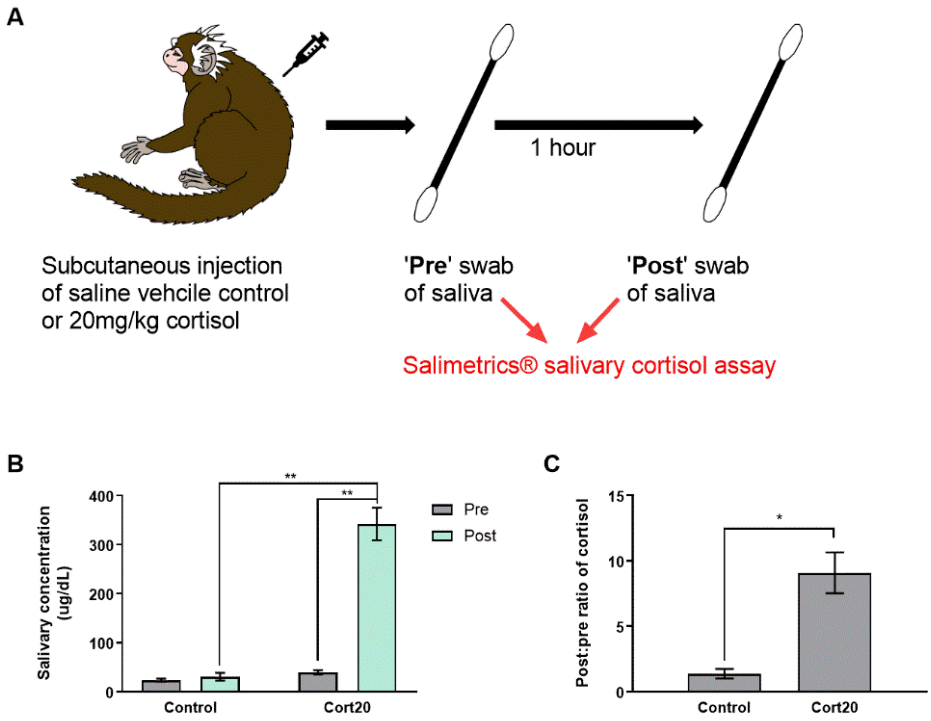


Figure 6-3 Subcutaneous cortisol injections successfully raise peripheral cortisol levels as measured by increases in salivary cortisol concentrations. Relevant graphs show mean ± SEM. N=4. **A** Four marmosets received a subcutaneous injection of saline vehicle control or 20mg/kg cortisol, followed by retrieval or a 'pre' swab of saliva immediately after injection. A 'post' swab was taken 1 hour later, consistent with the wait time used in manipulations on subsequent behavioural paradigms. The 'pre' and 'post' swabs were analysed using the Salimetrics® salivary cortisol assay as described in **2.5 Salivary Cortisol Sampling**. **B** Subcutaneous injections of 20mg/kg cortisol increased salivary cortisol concentrations in a phase-dependent manner (manipulation × phase, $F_{1,3}$=50.77, p=0.006) – there was no difference in salivary cortisol concentrations in 'pre' samples taken under cortisol *vs.* control conditions, but there was a significant increase in salivary cortisol concentrations in 'post' samples (effect of manipulation: 'pre,' p=0.852; 'post,' p=0.004). Furthermore, whilst there was no difference in 'post' *vs.* 'pre' samples under control conditions, cortisol levels were significantly higher in the 'post' *vs.* 'pre' sample following cortisol administration (effect of phase: control, p=0.969; cort20, p=0.004). **C** The ratio of 'post':'pre' salivary cortisol concentrations was significantly higher in the case of cortisol administration (two tailed paired *t*-test, p=0.023).

In **Chapter 5**, sgACC/25 over-activation was reported to elevate salivary cortisol levels following extinction. The mean levels of cortisol in the 'post' measurement of this study was 818 ± 186 nmol/L, meaning that the levels of cortisol obtained here were an order of magnitude higher. Whilst the results presented below nonetheless provide insight into whether elevations in HPA axis output *can* induce a similar array of changes to sgACC/25

over-activation, the actual contribution of increases in levels of cortisol to the over-activation induced phenotype will likely be subtler than the magnitude of effects observed here.

6.4.2 Subcutaneous cortisol injections induce behavioural – but not cardiovascular – signs of anticipatory anhedonia, without affecting reward consumption

At every dose tested (5mg/kg, 20mg/kg and 40mg/kg), injections of cortisol did not affect CS+ induced anticipatory MAP responses (**FIGURE 6-4A**) but did significantly blunt anticipatory behavioural arousal, as indexed by a reduced number of head-jerks during the CS+ (**FIGURE 6-4B**). Consummatory cardiovascular arousal to the US+ was also unaffected (**FIGURE 6-4C**), although the US responses in this cohort were significantly more variable than those observed in the cohort described in **Chapter 4**: indeed, only two out of four animals showed consistent US directed arousal responses under control (subcutaneous saline) conditions. Nevertheless, behaviourally, animals consumed a consistent amount of food reward, and this amount was also unaffected at any cortisol dose (**FIGURE 6-4D**). These data indicate that subcutaneous injections of cortisol induce behavioural signs of anticipatory anhedonia but do not affect cardiovascular anticipatory arousal, and further, they do not reduce consummatory arousal.

We also assessed the consummatory profile of animals receiving cortisol injections in a manner directly comparable to rodent studies, using the sucrose preference test adapted for marmosets (as described in **Chapter 4**). In the session prior to manipulations, this cohort of marmosets also showed a high preference for sucrose solution over water and consumed large amounts of sucrose in both the first 30 minutes (**FIGURE 6-5A**) and across the two-hour testing window (**FIGURE 6-5B**). Subcutaneous injections of 20mg/kg cortisol had no effect on sucrose preference (**FIGURE 6-5C**) or consumption (**FIGURE 6-5D**) in the first 30 minutes, nor did they have any effect on these measures across the two-hour session (**FIGURE 6-5E**), demonstrating that there is no obvious effect of this dose on reward consumption.

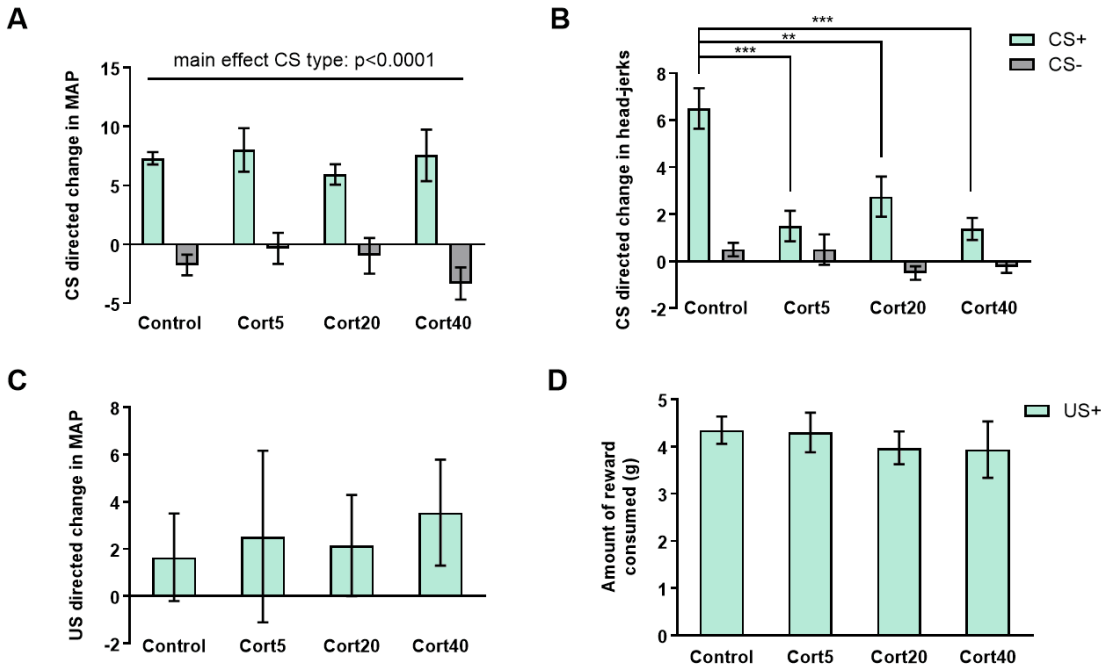


Figure 6-4 Subcutaneous cortisol injections induce behavioural – but not cardiovascular – signs of anticipatory anhedonia, without affecting reward consumption. Relevant graphs show mean ± SEM. N=4. **A** There was no effect of any dose of cortisol on anticipatory CS directed cardiovascular (MAP) arousal (manipulation × CS, $F<1$, NS; main effect of CS maintained, $F_{1,24}=77.67$, $p<0.0001$). **B** Cortisol manipulations blunted anticipatory behavioural arousal in a CS dependent manner (manipulation × CS, $F_{3,9}=9.50$, $p=0.004$), reducing head-jerks to the CS+ at every dose (effect of manipulation on CS+: cort5, $p<0.001$; cort20, $p=0.002$; cort40, $p<0.001$) without affecting responses to the CS- (effect of manipulation on CS-: cort5, $p>0.999$; cort20, $p=0.490$; cort40, $p=0.696$). **C** Cortisol manipulations did not affect US directed cardiovascular arousal (effect of manipulation, $F<1$, NS) although US directed cardiovascular arousal was unreliable in this cohort (for example, the US directed response under control conditions did not significantly differ from 0: one-sample *t*-test compared to 0, $p=0.439$) which confounds interpretation. **D** The amount of reward consumed did not differ at any dose (effect of manipulation, $F<1$, NS).

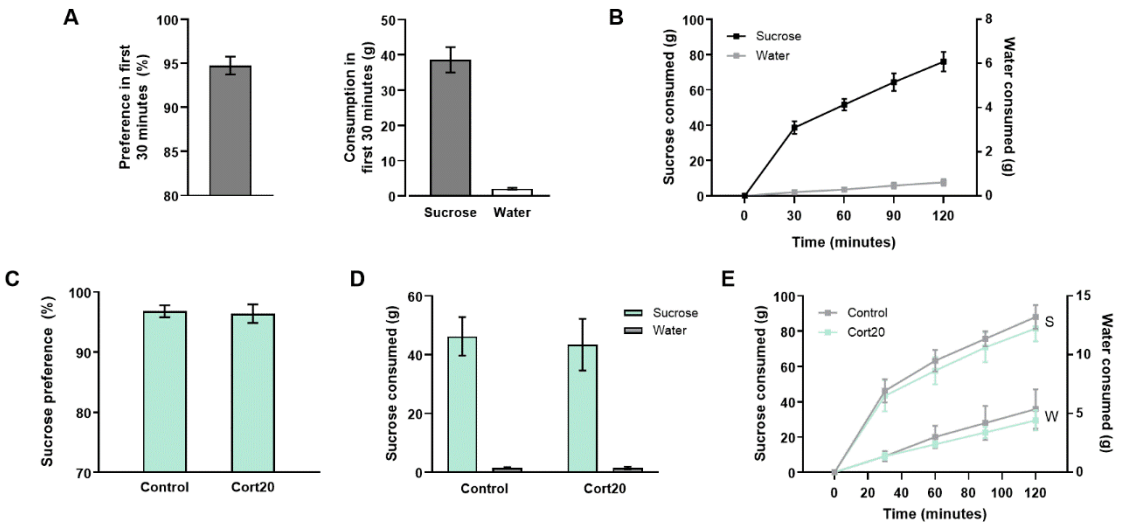


Figure 6-5 Subcutaneous cortisol injections do not affect reward consumption as measured in the sucrose preference test. Relevant graphs show mean ± SEM. N=4. **A** Prior to experimental manipulations, marmosets showed a high preference for sucrose during the first 30 minutes of the session (94.8 ± 1.0%), consuming 38.6 ± 3.6g sucrose and 1.3 ± 0.3g water (mean ± SEM). **B** Cumulative consumption profile in the session prior to experimental manipulations. Marmosets consumed significantly more sucrose at every timepoint measured (solution [water, sucrose] × timepoint [four, 30-minute time-bins], $F_{3,9}$=69.85, p<0.0001; effect of solution, p<0.0001 at every timepoint). **C** 20mg/kg cortisol injections had no effect on sucrose preference in the first 30 minutes of the session (two-tailed paired *t*-test, p=0.809). **D** 20mg/kg cortisol injections had no effect on either sucrose or water consumption in the first 30 minutes of the session (solution × manipulation, $F_{1,3}$=1.26, p=0.344; main effect of manipulation, $F_{1,3}$=1.49, p=0.310). **M** Across the two-hour session, 20mg/kg cortisol injections had no effect on cumulative sucrose or water consumption (solution × manipulation, $F_{0.3903,1.717}$=5.22, p=0.178; main effect of manipulation, $F_{0.2844,0.8533}$=1.33, p=0.302).

6.4.3 Subcutaneous cortisol injections increased anxiety scores in response to a HI

Compared to injections of saline vehicle control, all four marmosets showed increased anxiety following 20mg/kg cortisol injection as measured by an increased EFA-derived anxiety score in response to the HI (**FIGURE 6-6A**). The effect was not as strong as that observed with over-activation of sgACC/25: here, the mean ± SEM difference in anxiety score associated with cortisol was 0.49 ± 0.14, whereas the difference in anxiety score associated with sgACC/25 over-activation was 0.83 ± 0.11 (**Chapter 5**). It is worth noting that control levels of anxiety in this cohort (-1.26 ± 0.36) were, in general, lower compared to the control levels of anxiety measured in **Chapter 5** (-0.32 ± 0.24). This could contribute to the difference in effect magnitude. For instance, if the effects of anxiogenic agents depend on a

certain baseline level of threat perception, it could be expected that the size of effect is smaller as the cohort were not as anxious in control conditions.

The increase in anxiety score induced by cortisol was driven by a marked reduction in the TSAF (**FIGURE 6-6B**) (-21.4 ± 2%). There was also a significant increase in the number of tse calls (**FIGURE 6-6I**) – however, the increase was small (2.3 ± 0.5 calls) and this measure does not significantly load onto the anxiety score (and therefore does not contribute). Other measures were more variable and did not show significant increases/decreases across the cohort (**FIGURE 6-6C-H, J, K**).

The responses of animals across control and cortisol conditions are shown in **TABLE 6-1**.

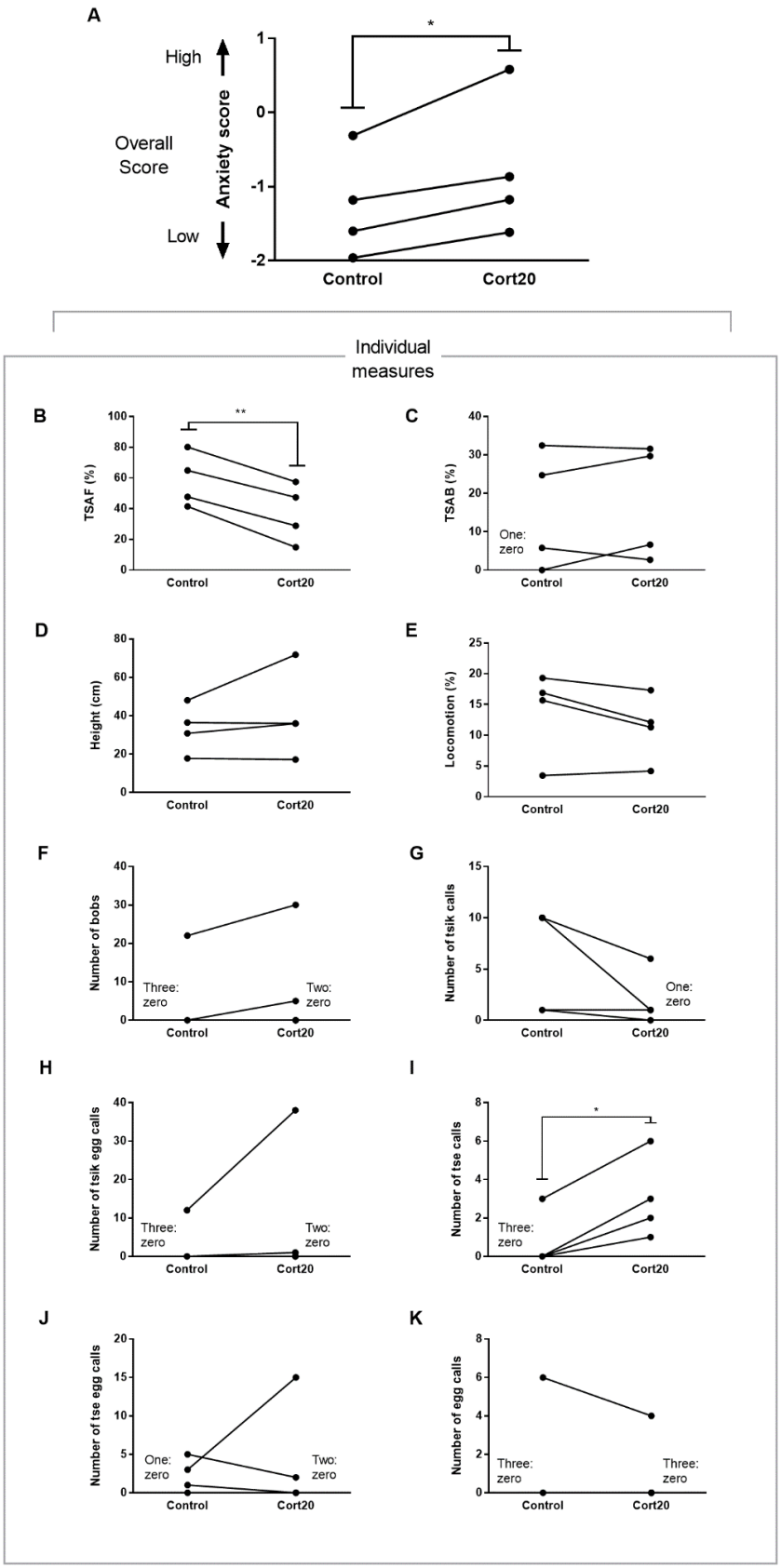


Figure 6-6 Subcutaneous cortisol injections increase anxiety responses to an HI.

Overall anxiety score shown top, with individual measures below. P values reported from two-tailed paired *t*-tests. N=4. **A** 20mg/kg cortisol injections increased EFA-derived anxiety scores (p=0.035). **B** Time spent at front (TSAF, %; p=0.002). **C** Time spent at back (TSAB, %; p=0.472). **D** Height (cm; p=0.315). **E** Locomotion (%; p=0.133). **F** Number of bobs (count; p=0.198). **G** Number of tsik calls (count; p=0.182). **H** Number of tsik egg calls (count; p=0.370). **I** Number of tse calls (count; p=0.018). **J** Number of tse egg calls (count; p=0.597). **K** Number of egg calls (count; p=0.391).

Subject/manipulation	EFA	TSAF, %	TSAB, %	Height, cm	Locm., %	Bobs	Tsik	Tsik-egg	Tse	Tse-egg	Egg
Subject 9											
Sal(Cort)	-1.18	0.48	0.25	36.46	16.89	0	10	0	3	1	0
Cort20	-0.87	0.29	0.30	35.99	12.11	0	1	0	6	0	0
Subject 17											
Sal(Cort)	-1.60	0.65	0.00	30.82	19.3	0	1	0	0	0	0
Cort20	-1.18	0.47	0.07	35.89	17.33	5	1	1	1	0	0
Subject 18											
Sal(Cort)	-1.96	0.80	0.06	17.75	15.68	0	1	0	0	3	0
Cort20	-1.62	0.57	0.03	17.17	11.3	0	0	0	3	15	0
Subject 19											
Sal(Cort)	-0.31	0.41	0.32	48.01	3.45	22	10	12	0	5	6
Cort20	0.58	0.15	0.32	71.79	4.17	30	6	38	2	2	4

Table 6-1 HI behaviours for all four subjects for control and 20mg/kg cortisol conditions. Subject numbers correspond to those outlined in **TABLE 2-3** (cohort three).

6.5 DISCUSSION

The work presented here is the first to describe the effects of acute peripheral cortisol injections on reward-related and anxiety-related behaviours in NHPs. Subcutaneous injections of cortisol successfully increased peripheral levels of cortisol as measured by increases in salivary cortisol concentration, confirming the manipulation has the desired effect. The cortisol concentrations achieved were consistent with peak circadian levels and levels associated with stress exposure but were significantly higher than those associated with sgACC/25 over-activation. Cortisol administration blunted behavioural (but not cardiovascular) signs of anticipatory arousal without any effect on reward consumption, and increased intolerance of uncertainty as measured by increased anxiety scores to the HI.

6.5.1 Blunted anticipatory but intact consummatory appetitive arousal following acute cortisol administration

In this chapter, the effects of acute cortisol administration to selectively blunt the behavioural aspects of anticipatory reward processing, without affecting consummatory reward arousal, have been demonstrated for the first time. Existing literature in rodents suggests that cortisol can actually *increase* reward anticipation/motivation by promoting DA release in the ventral striatum (Piazza and Le Moal, 1997). However, evidence in humans is contrary to this: acute stress appears to attenuate reward sensitivity by blunting signalling activity in the striatum and in PFC subregions including dACC (Berghorst et al., 2013; Ossewaarde et al., 2011). Montoya *et al.* examined the effect of acute cortisol administration on striatal and amygdala activation during the anticipatory phase of the MID task (see **FIGURE 1-26**) (Montoya et al., 2014). This study found that cortisol strongly decreased activity of both regions during anticipation of reward. Importantly, this downregulation was associated with subjective changes, with subjects receiving cortisol reporting significantly reduced reward preference.

The effect of acute and chronic stress on reward consumption has been extensively studied in the context of feeding behaviours (Yau and Potenza, 2013). It has been appreciated for some time that stress can have both activating and inhibiting effects on consummatory behaviour (inducing hyperphagia or hypophagia) (Levine and Billington, 1989). On the one hand, acute stress induces changes in behavioural, autonomic and endocrine functions which promote fight-or-flight reactions, and activities that may conflict with fight-or-flight behaviours are usually inhibited, including feeding. However, *chronic* stress may have opposing effects to stimulate eating (Gibson, 2006). Very few studies have assessed the effects of acute cortisol on the hedonic, subjective aspect of reward consumption, although data from one study suggest that acute cortisol increases the hedonic value of highly palatable food-related stimuli whilst simultaneously reducing their incentive motivational value (Ferreira de Sá et al., 2014).

The fractionated effect of cortisol on anticipatory, but not consummatory, arousal has relevance both (i) to the phenotype observed following sgACC/25 manipulations in **Chapter 4** and (ii) to the phenotype observed in depressed patients, who predominantly show impairments in anticipatory and motivational domains rather than consummatory domains (Der-Avakian and Markou, 2012; Treadway and Zald, 2011). Data already suggest a link between symptoms of anhedonia, disrupted cortisol dynamics and alterations in vmPFC activity. Dysfunctional cortisol responses (including elevated levels of morning cortisol) have been linked to higher levels of anhedonia seen in post-stroke patients with depressive symptoms (Terroni et al., 2015), and anhedonic symptoms have been linked to dysfunctional regulation of the stress axis by sgACC/25 (PUTNAM et al., 2008). Note that these studies used 'classic' questionnaire-based assessments of anhedonia, which fail to account for differences in reward anticipation and reward consumption. Further work is required to ascertain whether (acute or chronic) elevations in cortisol in humans can be associated with selective effects on reward anticipation *vs.* reward consumption, and whether these are linked to vmPFC-mediated regulation of the HPA axis.

Intriguingly, the effects we observed on the anticipatory phase of reward processing were selective to the behavioural domain – there were no effects on cardiovascular arousal. To date, no study has directly compared the effects of acute elevations of cortisol on behavioural and cardiovascular arousal associated with reward anticipation. Possible explanations for these differential effects include:

- The doses of cortisol used in this study are enough to blunt behavioural arousal, but not cardiovascular arousal; or
- Acute cortisol administration has a selective effect on brain regions involved in the behavioural but not cardiovascular responses to reward-predicting cues.

An uncoupling of autonomic and behavioural arousal has been associated with OFC lesions (Reekie et al., 2008), but has also been observed in patient groups with schizophrenia (Aleman and Kahn, 2005; Williams et al., 2007) and autism (Hirstein et al., 2001). Whether dysfunction in HPA axis regulation underlies this disjunction in physiological and behavioural function remains a possibility worth investigating, and whether this extends to patients with anxiety and depressive disorders remains unknown.

6.5.2 Elevated anxiety following acute cortisol administration

In addressing the association between cortisol levels and the expression of anxiety-related behaviours, two questions are immediately relevant:

- Do acute and/or chronic elevations of cortisol induced by drug treatment directly affect the expression of anxiety-related behaviours?

- Are levels of cortisol elevated – at baseline or in response to stressors – in populations with a higher anxious temperament and/or in patients with anxiety disorders?

The HI results presented in this chapter are most pertinent to the first question, where we have shown increased expression of anxiety-related behaviours following acute elevations in cortisol during exposure to an HI. Specifically, the HI test measures intolerance of uncertainty to an unfamiliar human. Whilst no studies to date have measured the effects of exogenous cortisol administration on intolerance of uncertainty, several rodent, NHP and human studies have assessed the effects of HPA axis manipulations on anxiety behaviours more generally. Acute elevations of CRH (which presumably increase cortisol levels) increase the expression of anxiety on an open-field test in rodents (Sutton et al., 1982), and chronic elevations in glucocorticoids sensitise similar behaviours (Rosen and Schulkin, 1998). Acute elevations in CRH also increase social-anxiety behaviours in NHPs (Strome et al., 2002). Comparatively few studies have assessed the effects of acute administration of exogenous glucocorticoids in humans, although those that have suggest variable effects: a dose-dependent effect to increase the startle response (Buchanan et al., 2001) but to reduce in phobic fear (Soravia et al., 2006).

Regarding the second question, higher basal and acute stress-induced cortisol levels have been observed in humans with higher trait anxiety levels (Brown et al., 1996; Takahashi et al., 2005). In patient populations, higher basal cortisol levels have been observed in panic disorder (Wedekind et al., 2000) and in PTSD (Yehuda, 1997) although these results are more variable (Young and Breslau, 2004). Furthermore, higher levels of subjective stress are associated with higher levels of intolerance of uncertainty in clinical settings (Kurita et al., 2013). These studies provide evidence for a link between subjective stress, the endocrine indices of stress and increased anxiety together with enhanced intolerance of uncertainty. The work presented in this chapter goes beyond correlation, implicating elevated levels of circulating cortisol as being causally responsible for this relationship.

Comparing the anxiogenic effects of cortisol to the anxiogenic effects of sgACC/25 over-activation reported in **Chapter 5**, it is apparent that the anxiogenic effect of cortisol is more limited. The increase in anxiety induced by cortisol is smaller and restricted to an effect on TSAF. By contrast, sgACC/25 over-activation impacts upon a range of measures to increase anxiety scores: TSAF, TSAB, height and locomotion. Given the potential effects of sgACC/25 over-activity on cortisol levels in aversive contexts, it is still possible that elevations in cortisol concentrations are responsible for some of the anxiogenic effects of sgACC/25 over-activation. However, the final phenotype appears likely to depend on factors not limited to elevations in cortisol (for example, effects on cardiovascular activity).

6.5.3 Contributions of elevated cortisol to the over-activation induced phenotype

In sum, these results indicate that elevated levels of circulating cortisol result in blunted behavioural – but not cardiovascular – appetitive arousal and moderate elevations in anxiety – in effect, a phenotype more limited in scope and magnitude compared to sgACC/25 over-activation. Furthermore, the level of cortisol elevation achieved in this chapter was significantly higher than the levels measured after over-activation during the Snake Extinction test (**Chapter 5**). Collectively, this would suggest that acute elevations in cortisol alone are unlikely to explain the anhedonia- and anxiety-like changes following over-activation. However, in a diseased state, elevations in cortisol are chronic and sustained over longer periods of time, as are the increases in sgACC/25 activity. In such situations, elevated cortisol levels may play a more substantial role.

6.5.4 Future work investigating the effects of chronic cortisol, and the action of cortisol in the context of the ventromedial prefrontal cortex

The characterisation of the effects of acute cortisol on reward- and anxiety-related behaviours serves predominantly as a foundation for future work (**FIGURE 6-7**). Firstly, it would be pertinent to determine the effects of cortisol manipulations on motivational processing in the progressive ratio paradigm, to ascertain whether elevations in cortisol reduce the motivation to work for reward. Secondly, it is important to establish the effects of chronic cortisol on the behavioural paradigms described in this chapter. The most translationally-relevant assessment of the effects of cortisol elevations on appetitive and aversive behaviours would involve cortisol administration over longer periods of time – several days to weeks. Longer administration periods would more closely mimic the chronic stress associated with pathological states observed in psychiatric conditions. Chronic delivery of cortisol could be achieved using osmotic minipumps (Alzet, Cupertino, CA). [I have consulted with manufacturers of these minipumps and established this a future line of investigation.]

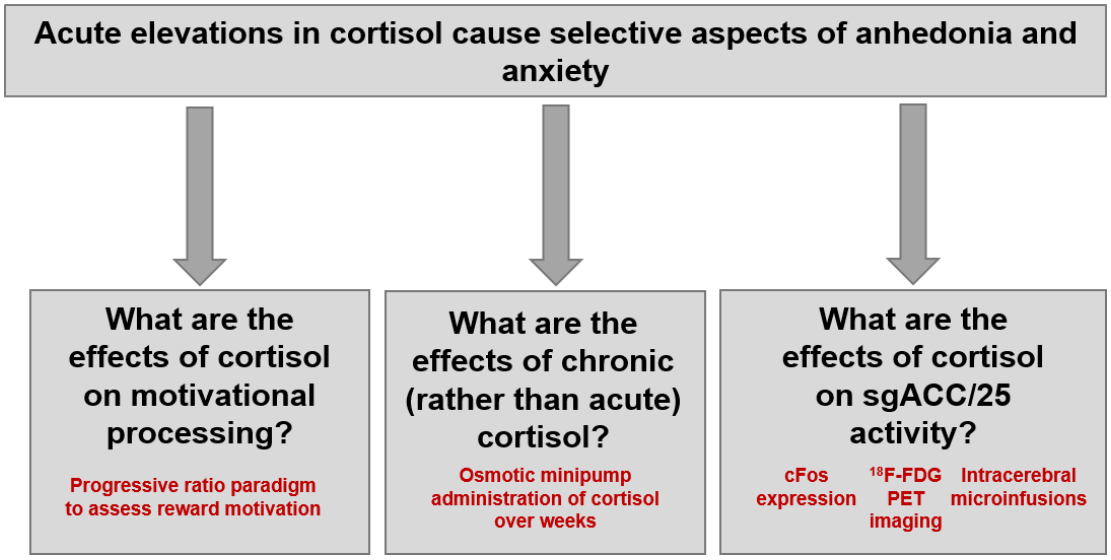


Figure 6-7 Future studies stimulated by the work presented in this chapter. The first set of studies concerns the effects of cortisol on motivational processing. The second set of studies concerns the effects of chronic administration of cortisol. The third set of studies concerns the interaction between elevated peripheral cortisol levels and activity within sectors of NHP vmPFC – particularly sgACC/25. See text for further details.

Thirdly, the interaction between sgACC/25 activity and cortisol levels warrants further investigation. This thesis has already discussed the effects of sgACC/25 over-activity on salivary cortisol levels – whilst over-activation of sgACC/25 does not appear to impact on cortisol levels in neutral conditions, it does appear to elevate cortisol levels following exposure to an aversive context. This suggests a causal influence of sgACC/25 activity on HPA axis regulation during situations of sustained contextual threat. However, the effects of cortisol on vmPFC activity (the 'other direction') remain unclear, together with precisely how cortisol interacts with the vmPFC more generally. These issues could be tackled with several approaches:

- **cFos expression**. The effects of peripheral cortisol administration on brain-wide cFos expression could be assessed. In rodents, Koot and colleagues have shown that acute cortisol injections increase cFos expression in IL (the putative homologue of sgACC/25) (Koot et al., 2013).
- **^{18}F-FDG imaging**. Cortisol injections could be performed in the context of ^{18}F-FDG PET imaging to determine the effects on regional cerebral metabolism. The advantage of this approach would be the ability to carry out three scans: a control scan, a scan following acute cortisol treatment and a scan following chronic cortisol treatment.

- **Infusions of cortisol into sgACC/25.** Cortisol infusions into IL have been shown to have deleterious effects on decision making performance in rodents (Koot et al., 2014). Using the behavioural paradigms described above, the importance of sgACC/25 corticosteroid signalling could be assessed on appetitive/aversive processing. This approach would also be amenable to ^{18}F-FDG PET imaging to determine the downstream consequences of increasing cortisol levels in sgACC/25.

These methods would proffer further insight into the precise nature of the interaction between peripheral stress hormone levels and activity within the vmPFC. Indeed, the behavioural impacts of elevated peripheral stress hormone levels may be mediated – at least in part – by central effects to alter activity in vmPFC subregions, given that several studies have shown that NHP vmPFC has particularly high concentrations of GCRs and MCRs (Patel et al., 2000; Sánchez et al., 2000). Should a link be definitively established between vmPFC activity and HPA axis regulation – together with their dysfunction in mood and anxiety disorders – this may represent a pathway for targeted treatment, centred on normalising dysfunctional neural and endocrine responses to stress. It may also contribute to our mechanistic understanding of how altered activity in ventromedial subregions so often seen in psychiatric disorders is causally related to specific symptoms.

6.6 Conclusion

The results presented in this chapter describe the effects of acute doses of cortisol to elevate anxiety and induce behavioural signs of anticipatory anhedonia, with no effect on reward consumption. These findings resemble in part the changes induced by sgACC/25 over-activation, although the concentrations of cortisol involved were significantly higher. Primarily, these data serve as a foundation for several avenues of future work – including, but not limited to, the effects of cortisol on reward motivation; the chronic effects of cortisol; the neural correlates of elevated levels of stress; and the interaction between HPA axis activity, stress and subregions of the vmPFC – particularly sgACC/25. Detailed characterisation of the interactions between peripheral cortisol, vmPFC activity and HPA axis output could be invaluable in deepening our understanding of the pathophysiology and aetiology of mood/anxiety disorders, together with revealing novel therapeutic targets.

7 General Discussion

Abbreviation	Meaning
^{18}F-FDG PET	^{18}Fluorine-fluorodeoxyglucose positron emission tomography
AAV	Adeno-associated virus
ANS	Autonomic nervous system
BAS	Behavioural activation system
BIS	Behavioural inhibition system
BNST	Bed nucleus of the stria terminalis
CaMKIIa	Calcium/calmodulin-dependent protein kinase IIa promoter
CBT	Cognitive behavioural therapy
CNO	Clozapine-*N*-oxide
CNS	Central nervous system
CS	Conditioned stimulus
CSI	Cardiac sympathetic index
CVI	Cardiac vagal index
dACC	Dorsal anterior cingulate cortex
DHK	Dihydrokainic acid
DMN	Default mode network
dmPFC	Dorsomedial prefrontal cortex
DREADD	Designer receptor exclusively activated by designer drug
EAAT2	Excitatory amino acid transporter-2
GABA	*γ*-aminobutyric acid
GAD	Generalised anxiety disorder
GPCR	G-protein coupled receptor
HI	Human intruder
HPA	Hypothalamo-pituitary adrenal
HR	Heart rate
HRV	Heart rate variability
IL	Infralimbic (cortex)
IU	Intolerance of uncertainty
MAP	Mean arterial pressure
MI	Myocardial infarction
mPFC	Medial prefrontal cortex
NHP	Non-human primate
NMDA	*N*-methyl-*D*-aspartate (receptor)
pgACC	Perigenual anterior cingulate cortex
PL	Prelimbic (cortex)
SAD	Social anxiety disorder
sgACC	Subgenual anterior cingulate cortex
siRNA	Short inhibitory ribonucleic acid
SSRI	Selective serotonin reuptake inhibitor
US	Unconditioned stimulus
vmPFC	Ventromedial prefrontal cortex

For decades, the neural circuitry underlying symptoms of mood and anxiety disorders has remained a black box. Whilst correlative human neuroimaging studies have implicated over-activity in the highly heterogeneous vmPFC, including sgACC/25 and pgACC/32 (Drevets et al., 2008a; Mayberg, 1997; Mayberg et al., 2005), we know nothing about the causal role of these areas in specific symptoms. Findings from interventional studies in rodents are extremely important, but their translational potential is limited since the comparability of rodent vmPFC subregions to those of NHPs and humans is unclear. The community at large cites anatomical homology as meaning functional analogy, but whether this is the case is far from clear (see (Myers-Schulz and Koenigs, 2012) and (Wallis et al., 2017)).

Despite the dearth of knowledge, to date there have been no studies investigating the consequences of over-activity of vmPFC subregions in monkeys. Elucidating the prefrontal contributions to symptoms of psychiatric disorders is of major translational importance. It is through an understanding of such neural changes that we can begin to appreciate why certain treatments are effective, and importantly, to facilitate the development of new treatment strategies. Novel therapeutic strategies are needed, with recent estimates placing mental illness as the leading cause of global burden of disease as measured by years lived with disability (Vigo et al., 2016). Interventional manipulation studies in NHPs are essential if we are to determine the causal neurobiological mechanisms underlying the symptom clusters constituting these disorders and how these symptoms respond to treatments.

The work in this thesis has made several inroads into characterising the autonomic, endocrine and behavioural features of over-activity within specific subregions of marmoset vmPFC: sgACC/25 and pgACC/32. The methodologies employed in the experiments described also illustrate broader considerations when researching psychiatric disorders. First, that seemingly unitary clinical symptoms (such as anhedonia and enhanced negative emotion) consist of distinct subtypes, which have different neurobiological bases. Second, that caution is required when inferring causality from strictly correlative neuroimaging studies: for example, whilst dysfunctional activity in a region encompassing pgACC/32 has been implicated in impaired reward processing, we have demonstrated that such changes are not causally related to impairments in anticipatory or consummatory arousal. Third, the utility of employing behavioural, autonomic and endocrine measures of emotion within a single study is highlighted, providing an unrivalled opportunity to quantify affect and bridge the gap between studies in humans (which often use physiological measures) and rodents (which frequently assess behaviour in isolation). Finally, the use of intracerebral microinfusions together with ^{18}F-FDG PET imaging represents a novel combination of technologies with immense utility, permitting the causal manipulation of brain regions and detailed characterisation of the downstream consequences associated with these manipulations.

7.1 Summary of Results

7.1.1 Peripheral physiological dysfunction associated with sgACC/25 over-activation

Correlative neuroimaging studies of patients with mood disorders have implicated over-activity within the vmPFC in mood disorders, and these same subregions are associated with the regulation of peripheral autonomic and endocrine function. Given the intimate association between physiological changes and psychiatric disorders (including depression and anxiety), an investigation of the causal consequences of manipulating these vmPFC subregions in NHPs is warranted. If activity changes within these regions are causally linked to cardiovascular/endocrine changes in a pattern like that observed in these conditions, this would reflect a critical neurobiological link in the association between mental illness and physiological dysfunction.

In **Chapter 3**, the cardiovascular and endocrine consequences of directly over-activating sgACC/25 and pgACC/32 in an emotionally 'neutral' condition are reported. Over-activation of pgACC/32 had no effect on peripheral cardiovascular function – however, the same manipulation of sgACC/25 had profound and extensive effects to elevate HR, reduce HRV and shift sympathetic:parasympathetic balance through a reduction in vagal tone. For the first time, sgACC/25 over-activity has been causally implicated in physiological changes which closely resemble those associated with a wide range of psychiatric disorders including depression (Brunoni et al., 2013; Carney et al., 2001; Stapelberg et al., 2012) and anxiety (Härter et al., 2003; Vogelzangs et al., 2010).

7.1.2 Fractionated anhedonia associated with sgACC/25 over-activation

A core feature of mood disorders is anhedonia – defined as a lack of ability to experience pleasure. The significance of reward-related deficits is becoming increasingly appreciated as both an important prognostic indicator and as a symptom that is resistant to current first-line therapies. The translational study of anhedonia has been hampered for several reasons: a lack of appreciation for the heterogeneous nature of the symptom (anticipatory, motivational and consummatory components); a fundamental mismatch between the constructs assessed in animal studies (consummatory – the sucrose preference test) and the patterns of impairments manifested in depressed patients (predominantly anticipatory and motivational); and a lack of clarity regarding the functional equivalence of human and rodent vmPFC subregions (Der-Avakian and Markou, 2012; Treadway and Zald, 2011, 2013).

In **Chapter 4**, the causal contributions of over-activity in sgACC/25, together with over-/under-activity in pgACC/32, were investigated with respect to specific subtypes of anhedonia. Whilst pgACC/32 manipulations had no effect, sgACC/25 over-activation blunted anticipatory and motivational arousal without affecting reward consumption – including no

effect on consumption as measured by the sucrose preference test. Not only do these results causally implicate sgACC/25 over-activation in a translationally-relevant anhedonic syndrome, but they emphasise the need for caution when interpreting the results of preclinical animal tests of symptoms of depression. Despite being considered the 'gold-standard' in preclinical assessment of anhedonia, the sucrose preference test is insensitive to profound deficits in reward processing which are not restricted to the consummatory domain. Using a novel combination of intracerebral microinfusions coupled with ^{18}F-FDG PET imaging, we provide evidence for downstream changes in interoceptive- and reward-related circuitry associated with sgACC/25 over-activation. These changes have implications for understanding the role sgACC/25 plays in the regulation of the behavioural and physiological aspects of emotion (see below).

7.1.3 Cardiovascular, behavioural and endocrine correlates of enhanced negative emotion associated with sgACC/25 over-activation

Negative emotions such as fear and anxiety are adaptive, multi-faceted mental states, emergent from a complex interaction between cognition, physiology and behaviour. If unregulated or inappropriately regulated, these responses can become maladaptive. From interventional studies in rodents, a causal role for sectors of the vmPFC – including both IL and PL – in regulating fear and anxiety has been revealed. However, the precise function of these regions is far from clear. For example, whilst augmenting the excitability of the putative sgACC/25 homologue, IL, strengthens extinction memories (Fontanez-Nuin et al., 2011; Milad and Quirk, 2012; Santini and Porter, 2010), the same manipulation increases innate anxiety behaviours (Bi et al., 2013). Beyond these seemingly contradictory functional findings, there remains uncertainty regarding the anatomical and functional similarity of IL to regions in the NHP and human. Indeed, recent work from the present author's laboratory has shown opposite effects on fear extinction associated with sgACC/25 inactivation (to enhance extinction), compared to those expected from the same manipulations of IL (to impair extinction) (Wallis et al., 2017).

In **Chapter 5**, we show for the first time that sgACC/25 over-activation in the NHP is causally related to both (i) elevated contextual arousal in aversive contexts and (ii) elevated anxiety associated with intolerance of uncertainty on the HI paradigm. We also found evidence to suggest that sgACC/25 over-activation is associated with impaired stress recovery and altered endocrine reactivity in these negative contexts. These results are consistent with a role for IL activity in promoting anxiety behaviours but are inconsistent with its putative role to enhance fear extinction. They further suggest a central role of sgACC/25 in coordinating behavioural, autonomic and endocrine aspects of anxiety responses in aversive contexts associated with the sustained potential for threat.

7.1.4 The novel antidepressant ketamine and its amelioration of sgACC/25 over-activation induced changes

The sequalae of sgACC/25 over-activation are translationally relevant to psychiatric disorders – the fractionated pattern of blunted arousal and motivation in appetitive contexts closely resembles anhedonia observed in depressed patients, and the elevated contextual arousal coupled with exaggerated endocrine reactivity resembles impairments in stress regulation associated with depression and anxiety. This offers an invaluable opportunity to test the efficacy of antidepressant agents on a transient, pharmacologically-induced state with face-validity where the cause is known. In so doing, insights can be gleaned into the specific symptoms for which these agents are effective treatments, together with the neurobiological basis of their efficacious action.

In **Chapter 4**, a single dose of the novel, glutamate-based antidepressant ketamine reverses anticipatory anhedonia induced by sgACC/25 over-activation in a time-dependent manner: whilst it fails to reverse impairments 4 hours after administration, it successfully reverses blunted reward arousal 1 day and 7 days later. The time course of ketamine's efficacy in this preparation closely matches the time course of clinical efficacy reported in clinical literature (Abdallah et al., 2015). By contrast, an acute dose of the antidepressant citalopram fails to reverse associated anhedonic impairments. In **Chapter 5**, the same dose of ketamine tested at a timepoint at which it showed efficacy in the anhedonic domain fails to reverse acutely elevated anxiety following sgACC/25 over-activation on the HI paradigm. Not only does this provide novel insight into the therapeutic profile of ketamine, it further suggests that different neurobiological substrates are at play in the changes induced by sgACC/25 over-activation.

7.1.5 Changes induced by peripheral injections of cortisol resemble, but do not mimic, changes induced by sgACC/25 over-activation

The vmPFC has been linked to HPA axis function in both rodents (Diorio et al., 1993; Loewy and Spyer, 1990; McKlveen et al., 2013) and primates (Jahn et al., 2010; Sudheimer et al., 2013). In **Chapter 3**, it was found that sgACC/25 over-activation has no effect on salivary cortisol levels in 'emotionally neutral' situations. However, in **Chapter 5**, sgACC/25 over-activity was found to elevate cortisol levels after exposure to an aversive context (associated with presentation of a rubber snake). In **Chapter 6**, we investigated whether artificially elevating cortisol levels could induce a similar array of changes to sgACC/25 over-activation to address whether such changes could, in part or in whole, account for the behavioural and physiological dysfunction induced by this manipulation.

20mg/kg cortisol injections acutely raised cortisol to levels equivalent to peak circadian values and values associated with stress exposure in marmosets (Ash et al., 2018), although

these levels were higher than those associated with sgACC/25 over-activation (**Chapter 5**). These elevated circulating cortisol levels resulted in blunted behavioural appetitive arousal, without affecting autonomic arousal and without affecting reward consumption. On the HI paradigm, elevations in cortisol resulted in moderately increased anxiety. These data indicate that whilst there are similarities between the cortisol-induced and over-activation induced phenotypes, in the appetitive setting the phenotype associated with cortisol injections was less extensive, and in the aversive setting it was both less extensive (affecting fewer behaviours on the HI test) and smaller in magnitude (despite the levels of circulating cortisol being higher). This suggests that even if cortisol is playing a role in the anhedonia and anxiety triggered by sgACC/25 over-activation, its role is comparatively limited over time-courses associated with pharmacological manipulations (acute/short-term). Whilst itself informative, the work presented in this chapter primarily serves as a foundation for future investigations addressing the consequences of *chronic* elevations in cortisol levels and *chronic* levels of over-activity in sgACC/25, together with studies aimed at dissecting the nature of sgACC/25-HPA axis interactions.

7.2 SYNTHESIS OF FINDINGS

7.2.1 Hypotheses regarding sgACC/25 function

As is summarized above, sgACC/25 over-activity is associated with a complex myriad of changes in behavioural and physiological domains. Although future work is critical, tentative accounts for the functions subserved by sgACC/25 can be suggested. I propose two main hypotheses below, to try and integrate the findings presented in this thesis and generate a parsimonious account of sgACC/25 function (**FIGURE 7-1**):

(i) **HYPOTHESIS 1: SgACC/25 is important in coordinating fight-or-flight reactions, through independent excitatory and inhibitory relationships with aversive- and appetitive-related arousal mechanisms, respectively** (**FIGURE 7-1A**). This explanation posits a direct excitatory relationship of sgACC/25 to structures stimulating aversive arousal together with a direct inhibitory/feed-forward inhibitory relationship to structures stimulating reward-related arousal/approach. One immediate issue with this model relates to the lack of effect of *inactivation* to elevate appetitive CS directed arousal (**Chapter 4**) and the lack of effect of *over-activation* to elevate aversive CS directed arousal (**Chapter 5**). However, these findings may be explained if (i) the levels of CS directed appetitive/aversive anticipatory arousal were already at ceiling levels under control conditions in these Pavlovian paradigms, or alternatively, (ii) if sgACC/25 is only activated in situations associated with aversive, but not appetitive, affective meaning – in which case inactivations would not impact upon appetitive Pavlovian arousal.

The hypothesized position of sgACC/25 in such a circuit would suggest that acute changes in its activity stimulate elevated negative arousal and withdrawal responses together with inhibition of aspects of reward related processing (diminished reward anticipation and motivation) impairing approach behaviour. These functions are ideally suited for a brain structure putatively involved in survival optimization during situations of threat (Mobbs et al., 2015). Furthermore, the baseline cardiovascular effects of sgACC/25 over-activation to diminish parasympathetic tone and thereby alter sympathetic:parasympathetic balance (**Chapter 5**) suggests that sgACC/25 is also causally related to the physiological changes necessary for a shift from ‘rest-and-digest’ (parasympathetic dominance) to ‘fight-or-flight’ (sympathetic dominance).

In proposing this hypothesis, I note two further considerations regarding the precise relationship sgACC/25 has to aversive- and appetitive- related arousal mechanisms in this scheme:

- Does elevated activity within sgACC/25 stimulate aversive arousal, and independently inhibit appetitive arousal?
- Does elevated activity within sgACC/25 stimulate aversive arousal which simply 'disrupts' appetitive arousal?

In the context of the latter explanation, sgACC/25 would not have a *functional* link (whether direct or indirect) to reward-related functions per-se. Instead, the effects on motivational and anticipatory reward processing would be a consequence of enhanced aversive-related behaviours. I find this account lacking for several reasons. First, if the effects on appetitive processing were solely related to enhanced negative emotion, over-activation associated impairments would be expected across all domains of reward processing including reward consumption. In **Chapter 4**, it was convincingly demonstrated that there is no effect on behavioural or autonomic measures of consummatory arousal. Second, there was no evidence of increased agitated behaviour associated with over-activation during appetitive Pavlovian, progressive ratio or sucrose preference paradigms. For example, locomotion scores during appetitive Pavlovian conditioning were no different from control infusions, suggesting that there was no freezing or hyperlocomotion in this context (both of which can be associated with anxiety). In addition, the reduced number of CS directed head-jerks has face validity to a state associated with reduced reward anticipation, and not a state of elevated anxiety. Third, the contrasting effects of ketamine on reward and anxiety-related dysfunction associated with over-activation imply different neurobiological mechanisms at play, since a dose of ketamine could restore appetitive behaviour in the absence of any change in anxiety behaviour as measured by the HI test (discussed in **Chapter 5**).

(ii) **HYPOTHESIS 2: SgACC/25 increases rumination and worry, manifesting as behavioural withdrawal** (**FIGURE 7-1B**). In proposing hypothesis 2, I am attempting to integrate findings presented within this thesis with existing literature, which consistently implicates sgACC/25 in '*negative-affect laden withdrawal*' associated with rumination (Bratman et al., 2015; Hamilton et al., 2015) together with activity in sgACC/25 associated with sustained threat (Alvarez et al., 2011; Hasler et al., 2007b, 2008). Rumination has been strongly linked to symptoms of both depression and anxiety (Michl et al., 2013; Verstraeten et al., 2011; Wilkinson et al., 2013) and in the context of anxiety disorders a closely related concept is termed 'worry.' Rumination and worry are frequently grouped together as reflecting patterns of *repetitive negative thinking* (Eysenck and Fajkowska,

2017; Topper et al., 2017), although the constructs are distinct: rumination is typically 'past-focused' whereas worry is 'future-focused' (Lewis et al., 2017).

A role for sgACC/25 in the *cognitive* operations of rumination and worry also links to the dominant biopsychological *behavioural* personality theory proposed by Gray positing separate Behavioural Inhibition and Behavioural Activation systems (BIS/BAS respectively) (Gray, 1987). Gray suggests that the BIS governs avoidance behaviour together with cognitive/behavioural withdrawal in response to threat/punishment, whereas the BAS regulates motivated and approach behaviour, reward seeking and positive affect. SgACC/25 has been most consistently implicated in inhibitory control functions similar to those proposed to be served by the BIS (namely, withdrawal) (Hamilton et al., 2015; Matthews et al., 2009). Nevertheless, some studies have also linked sgACC/25 to the BAS: *reduced* resting state functional connectivity of sgACC/25 to the DMN has been associated with *higher* BAS sensitivity and protective effects against excessive rumination (Iadipaolo et al., 2017). These data suggest that sgACC/25 activity is positively correlated with functions subserved by the BIS (withdrawal) and negatively correlated with functions subserved by the BAS (approach). Whether sgACC/25 has direct connectivity to the BAS independent of the BIS is not clear, but these two systems are proposed to mutually inhibit one another (Gray, 1987) such that an effect on one would invariably impact on the other, resulting in a *functional* link between sgACC/25 activity and *both* the BAS and the BIS.

If sgACC/25 increases rumination/worry and – because of an excessive focus on internally driven, self-referential processes – behavioural withdrawal, this may manifest as impaired responses to discrete appetitive cues in neutral/moderately positive contexts (**Chapter 4**) and exaggerated withdrawal (both behavioural and autonomic) in aversive contexts (**Chapter 5**). Consider the results observed on the HI paradigm: following sgACC/25 over-activation, animals showed increased depth and height in the cage together with reduced locomotion. Increased stillness, together with increased distance maintained from the intruder, is arguably face-valid with a state of behavioural 'withdrawal' rather than, for example, a state of panic.

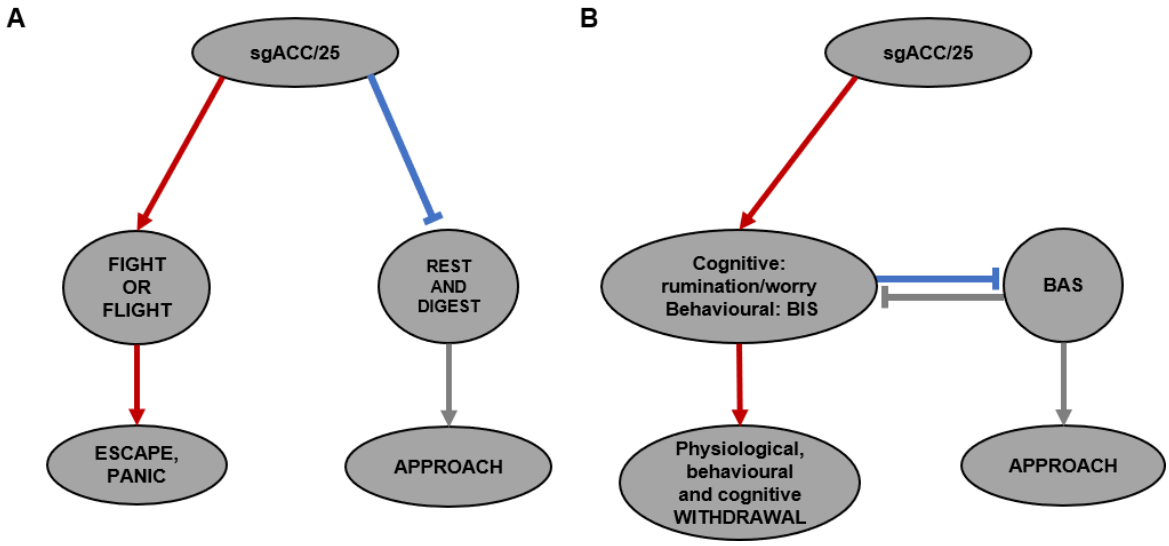


Figure 7-1 Hypotheses regarding sgACC/25 function based on data presented in this thesis. **A** SgACC/25 co-ordinates fight-or-flight responses, stimulating escape and panic responses and either inhibiting (shown here) or simply 'disrupting' rest-and-digest and appetitive behaviours. **B** SgACC/25 stimulates rumination/worry – or in Gray's behavioural framework, the BIS – promoting withdrawal and inhibiting approach through inhibition of the BAS. In this scheme, withdrawal is physiological, behavioural and cognitive, characterised by reduced parasympathetic tone (**Chapter 3**), impaired external 'cue-directed attention' and reduced motivation (**Chapter 4**), and increased distance/reduced locomotion ('stillness') in the HI test (**Chapter 5**).

Hypothesis 1 would – at a surface level – explain the results observed in this thesis. In this hypothesis, sgACC/25 is effectively conceptualised as having a key role in fight-or-flight responses: in such situations, it is appropriate to activate escape mechanisms and inhibit approach. However, in **Chapter 4**, there was no evidence that sgACC/25 triggered physiological or behavioural changes consistent with a fight-or-flight reaction in appetitive contexts. The pattern of results associated with sgACC/25 is more consistent – at a theoretical but also 'face' level – with hypothesis 2.

With relevance to both hypotheses, I note that the effects of sgACC/25 seem to be context-dependent. In **Chapter 5**, the increased cardiovascular and behavioural arousal associated with sgACC/25 over-activation is predominantly context-directed and was not observed when an identical manipulation was carried out in a neutral context. This would suggest that sgACC/25 has a contextual processing function. Furthermore, with regards to hypothesis 2, whether sgACC/25 triggers increased 'past-based' (rumination) or 'future-based' (worry) negative repetitive thinking patterns may depend on contextual information (neutral/appetitive *vs.* aversive contexts). Beyond the functional effects observed in **Chapter 5**, is there any further evidence for sgACC/25's involvement in contextual processing? In preliminary work,

we have carried out retrograde tracing studies targeting marmoset sgACC/25 to investigate afferent connectivity. In two animals, we have found extensive projections from the hippocampal formation (including subiculum, dentate gyrus, CA2 and CA1 subfields) to sgACC/25 (**FIGURE 7-2**). The hippocampal formation is frequently implicated in context processing and contextual fear conditioning (Alvarez et al., 2008; Ballesteros et al., 2014). Thus sgACC/25 appears to be ideally positioned to receive contextual information and use this to modulate its output.

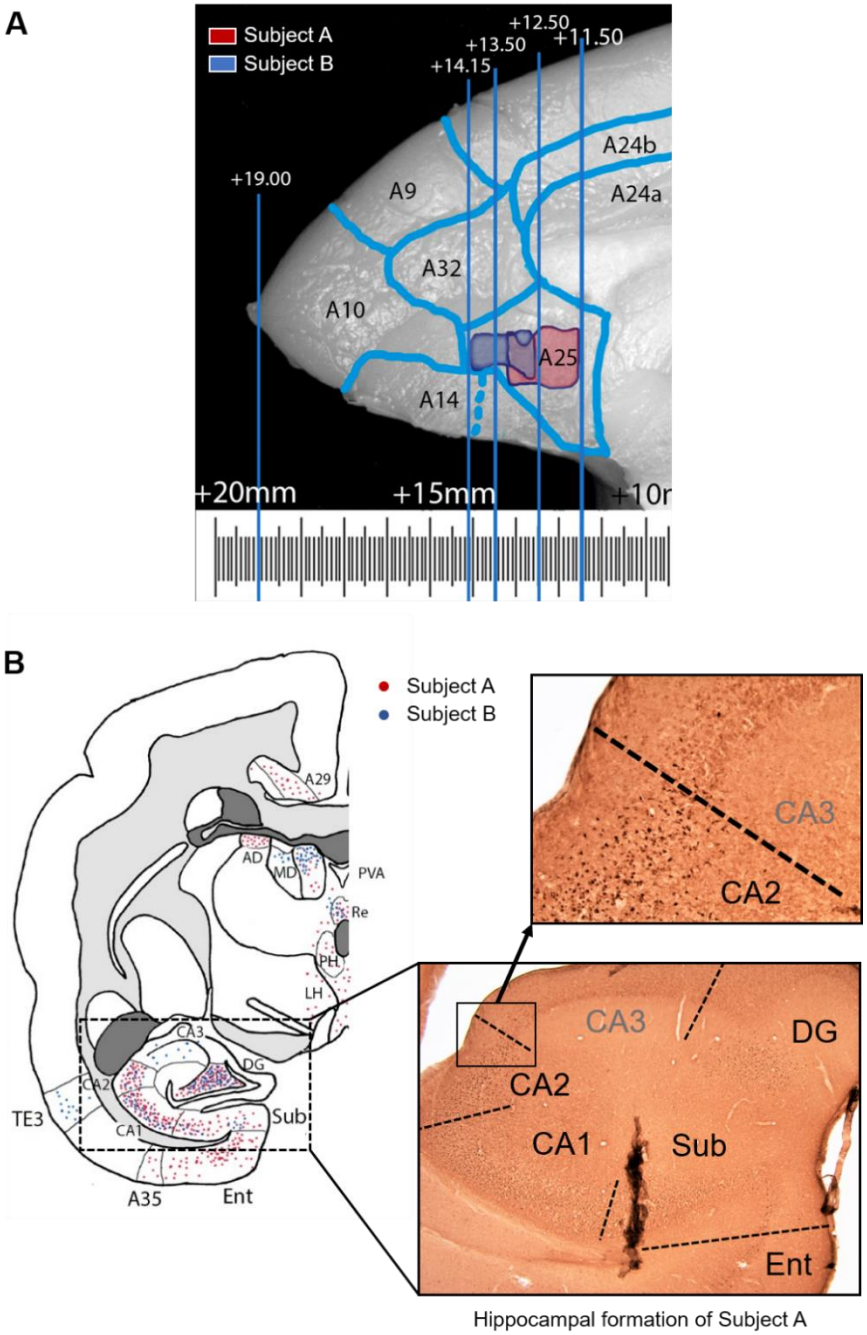


Figure 7-2 Afferent connectivity of sgACC/25: hippocampal formation. **A** In a stereotaxic surgical procedure, two animals (Subject A, red, and Subject B, blue) were infused with the

retrograde tracer cholera toxin subunit B into left sgACC/25. The sagittal section shows the AP spread of the tracer infusion within sgACC/25 (scale in mm from interaural line). **B** Ten days later animals were perfused, brains sectioned, and immunohistochemically stained cell bodies were mapped onto schematic sections of the marmoset brain. Left is a schematic section at the level of the hippocampal formation, showing extensive staining in the dentate gyrus (DG), subiculum (Sub) and CA2/CA1 cell fields in both subjects. Right is a photomicrograph of the hippocampal formation of subject A, with darkly stained bodies throughout the hippocampal formation with the notable exception of CA3. Inset shows the CA3/CA2 border zone.

How can hypothesis 2 be investigated further? Combining causal manipulations of sgACC/25 with imaging techniques facilitates investigation of downstream brain regions whose activity is changed by these manipulations. In **Chapter 4**, sgACC/25 over-activity in an appetitive context resulted in downstream hyperactivity in dmPFC, dACC and insula. An analogous region of dmPFC/dACC is posited as a key hub in the DMN of both humans (Raichle et al., 2001) and macaques (Mantini et al., 2011). Therefore, the downstream regions affected by sgACC/25 over-activation are themselves associated with internally-directed focus, and in the diseased state when this becomes excessive, rumination (Hamilton et al., 2015). This supports the suggestion that elevated activity in sgACC/25 may bias attention internally, rather than externally. In appetitive contexts, this may reduce the direction of attention to external cues which would otherwise act to elevate cardiovascular and behavioural arousal (**Chapter 4**). In aversive contexts, this may promote withdrawal (associated with 'worry'); a phenotype observed in the HI test (**Chapter 5**). Indeed, elevated connectivity between sgACC/25 and insula has been demonstrated during the induction of worry in elderly GAD patients (Andreescu et al., 2015).

The autonomic consequences of both sgACC/25 inactivation (to elevate vagal tone) (Wallis et al., 2017) and over-activation (to reduce vagal tone, **Chapter 3**) also lend further support to hypothesis 2. Cardiac vagal tone has been proposed to serve as an index of flexible, dynamic emotion regulation as part of polyvagal theory (Porges, 1992) and theoretical reviews by Thayer and colleagues have linked worry and rumination with inflexibility in the regulation of the cardiovascular system (Thayer and Siegle, 2002). Baseline vagal tone is a *'psychophysiological resource'* which organisms can mobilise to increase behavioural and cognitive flexibility. Low vagal tone has been specifically linked to rumination and worry, conceptualized as 'emotional inflexibility' (Pieper et al., 2007). Current theories posit that excessive rumination has the effect to release inhibitory prefrontal control over sympatho-excitatory neural circuits resulting in parasympathetic withdrawal, sympathetic dominance and impairments in adaptive behavioural responding (Brosschot et al., 2007; Ottaviani et al., 2009). Parasympathetic withdrawal (reduced CVI) and increased sympathetic dominance

(increased CSI:CVI ratio) were precisely the changes observed following sgACC/25 over-activation in **Chapter 3**.

The thesis has also presented data suggesting that sgACC/25 over-activity is linked to increased cortisol output in aversive contexts (**Chapter 5**). Is there a link between the HPA axis and excessive rumination/worry? In both a patient cohort with SAD and in healthy controls, Lewis and colleagues found that state/trait levels of worry (but not rumination) were associated with increased cortisol reactivity during a social stress test, whereas levels of *both* rumination and worry were associated with elevated cortisol levels during recovery (especially in SAD patients) (Lewis et al., 2017). Therefore, the physiological consequences associated with sgACC/25 over-activation – both autonomic and endocrine – mirror those associated with rumination and worry in humans.

The idea that elevated activity in sgACC/25 leads to an inflexible, internally-directed state relates closely to the hypothesis of Holtzheimer and Mayberg, who propose that depression is characterized by a tendency to enter into – but also *an inability to shift out of* – depressed mood states, rather than the presence of a state of low mood per-se (**FIGURE 7-3**):

> *"…we conceptualize the **depressive state** as an aberrant neural rhythm, but the **depressive disorder** as the brain's tendency to go into and stay in that rhythm inappropriately."* (Holtzheimer and Mayberg, 2011)

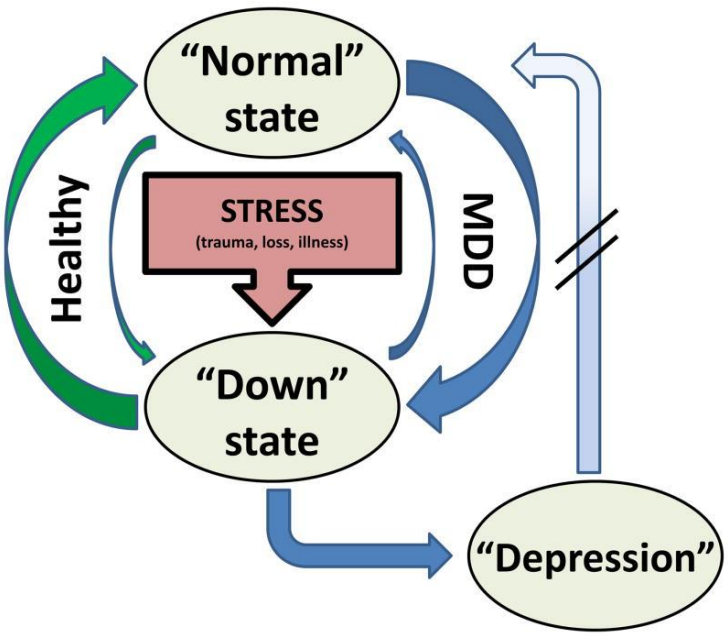


Figure 7-3 'Stuck-in-a-rut': depression as an inability to disengage from a negative mood state. Figure taken from Holtzheimer and Mayberg, 2011. In their model, a stressor triggers and shift from a euthymic "normal" state to a "down" state, characterised by symptoms of enhanced negative affect, anhedonia and sickness behaviour. In healthy individuals, the tendency to enter the

down state is low and the return to euthymia is quick (green arrows). In individuals with MDD, they have a high tendency to enter the down state and an impaired ability to re-enter the normal state (blue arrows). When in the down state – a depressive episode – patients may have an even greater difficulty returning to normal without psychotherapeutic, pharmacological or more invasive treatments. Holtzheimer and Mayberg emphasise that the down state *"itself is not abnormal… it is the tendency to enter and get stuck in this state that defines depression*."

The idea that depressed patients are 'stuck-in-a-rut' is germane with the hypothesis that depression and mood disorders are – at least in part – disorders of emotional inflexibility, elevated rumination and aberrantly increased levels of self-directed thought. SgACC/25 may represent a critical node in the depression network, associated with an increased tendency to enter a down state and an impaired ability to exit it. It is worth noting that Holtzheimer and Mayberg suggest that the neurobiology of depression should be that of '*mood reaction and regulation, rather than mood state*,' and that a structure involved in mood reaction would need to process external and internal stimuli with reference to contextual information.

In the context of hypothesis (ii), I draw further attention to the finding that sgACC/25 over-activity increases activity in the insula in appetitive settings (**Chapter 4**). The consequences of this hyperactivity in the network of brain regions whose activity is altered following sgACC/25 over-activation is not entirely clear and warrants further investigation. The insula forms a critical part of the salience network (Heine et al., 2012), involved in the orientation towards salient emotional stimuli and switching between self-referential (DMN) and task-relevant states (Seeley et al., 2007). The insula is also frequently implicated in interoceptive self-focus (Paulus and Stein, 2006). Collectively, these functions imply that the insula serves an integrative function, processing both internal and external signals to guide behaviour. I suggest three explanations for elevated activity in the insula:

(i) **Activity in the insula represents an error signal**. SgACC/25 over-activation in an appetitive context may induce a discrepancy between external (in this case, appetitive) and internal states (biased towards withdrawal and negative affect following sgACC/25 over-activation).

(ii) **Activity in the insula represents a compensatory change**. If sgACC/25 over-activation biases processing towards self-referential functions, the function of the insula as part of the salience network may try to compensate and direct attention in a task-dependent manner, towards motivationally relevant external stimuli.

(iii) **Activity in the insula reflects its function in visceral sensation**. Given that sgACC/25 over-activation induces changes in cardiovascular reactivity, the activity changes observed in the insula might reflect its function as 'visceral

sensory cortex,' a function proposed by Neafsey and colleagues (Neafsey et al., 1993).

To test explanation (i), future work investigating the downstream neural consequences of sgACC/25 over-activation in an aversive setting may provide insight (e.g. ^{18}F-FDG PET imaging combined with aversive behavioural testing and sgACC/25 over-activation – already under progress). Specifically, would regions such as the insula still be engaged when the context and internal state are more congruent (both putatively 'negative')? If not, this would support explanation (i). Explanation (ii) could be tested by causally manipulating the insula with intracerebral microinfusions (or other techniques), to determine if the changes in appetitive/aversive contexts are in any way like those associated with sgACC/25 over-activation. Finally, I would suggest that explanation (iii) is lacking – that the insula is simply sensing peripheral changes induced by sgACC/25 over-activation. Whilst this function of the insula may be partly responsible for the change in activity, I do not think it is the only one. Again, from preliminary tracing experiments there is evidence of both afferent *and* efferent connectivity between sgACC/25 and sectors of the insula (both rostral and caudal sectors) (**FIGURE 7-4**). This suggests that sgACC/25 and rostral/caudal insula are communicating in some way, although of course it does not give insight into the functional correlates of this cross-talk.

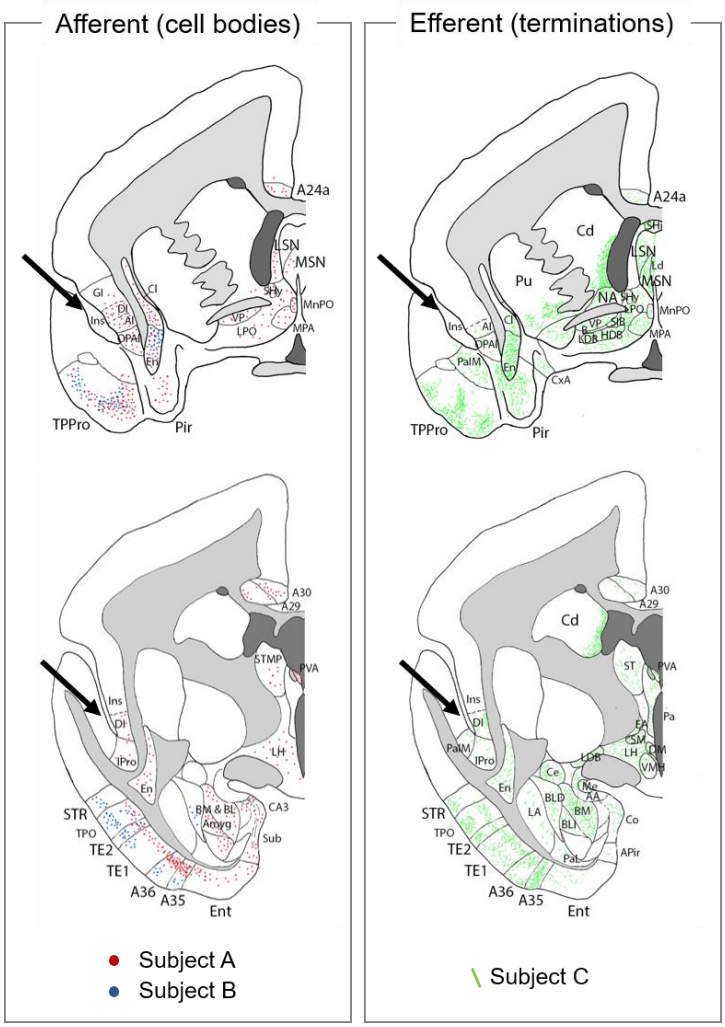


Figure 7-4 Afferent (left) and efferent (right) connectivity of sgACC/25: rostral (top) and caudal (bottom) insula. Subjects A (red) and B (blue) as described in **FIGURE 7-2**; Subject C (green) also underwent a stereotaxic surgery but was infused with the anterograde tracer biotinylated dextran amine, to investigate efferent connectivity. In each schematic drawing, the insula is indicated with a black arrow. In Subject A – but less so in subject B – there was evidence of afferent connectivity from the insula to sgACC/25. In Subject C, there was evidence of relatively extensive terminations in both rostral and caudal insula, illustrating that efferents from sgACC/25 terminate along the rostro-caudal extent of the insula. Relevant abbreviations: Ins, insula; GI, gustatory insula; DI, dysgranular insula; AI, agranular insula; OPAI, orbital periallocortex (a 'peri-insular' region); IPro, insular proisocortex.

The insula is a heterogeneous brain region, consisting of a rostral/anterior and caudal/posterior division which themselves are constituted of several subregions: for example, the anterior insula consists of the gustatory insula, dysgranular insula and agranular insula. The tracing data presented in **FIGURE 7-4** suggest that sgACC/25 shows afferent and efferent connectivity with most of these subregions. Whether specific zones of the insula are important for the impairments in reward processing/elevated anxiety

associated with sgACC/25 over-activation certainly needs further investigation. Intriguingly, the potential link between insula and vmPFC extends beyond the 'affective' domain into the cognitive. Recent work in rodents has shown that lesions of the anterior insula reduce the development of compulsive habits (Belin-Rauscent et al., 2016), and relatedly, IL lesions prevent the development of habits after extended training (Killcross and Coutureau, 2003). The similarity in behavioural outcome following anterior insula and IL lesions further supports the suggestion that the functions of these regions are linked. Disconnection strategies may be invaluable in investigating the importance of IL-vmPFC connectivity in Pavlovian and instrumental behaviours.

Note that the principle manipulation employed in this thesis – pharmacological over-activation – does not explicitly confirm the necessity of a brain region in a cognitive/behavioural function, although it does provide insight into the operations in which the region may be involved. By contrast, silencing a brain region via inactivation proffers critical, causal information pertaining to functions in which that brain region is required. Work described in this thesis, together with previous work from this author's laboratory, has used pharmacological inactivations to glean insight into the operations performed by sgACC/25. Specifically, area 25 inactivations (i) enhance parasympathetic tone in neutral conditions (Wallis et al., 2017); (ii) reduce the anticipatory arousal in response to CSs predicting an aversive US, without affecting arousal to the US itself (Wallis et al., 2017); and (iii) do not impair anticipatory or consummatory arousal response during appetitive CS/US pairings (this thesis).

Therefore, whilst sgACC/25 is necessary for regulating resting cardiovascular function and elevating arousal associated with negative emotion expectation, it is not *required* for enhancing arousal associated with positive emotion expectation. Work from Rudebeck and colleagues corroborates this suggestion, showing that ablative lesions of macaque sgACC/25 do not disrupt CS induced autonomic arousal (measured by pupil diameter) during appetitive Pavlovian conditioning (Rudebeck et al., 2014). This study did, however, find that sgACC/25 lesions disrupt the maintenance of autonomic arousal when a trace interval was present between the CS and US period. Although the use of ablative lesion techniques limits interpretation of the findings (since the observed phenotype may result from damage to fibres of passage), the suggestion that sgACC/25 may be involved in sustaining autonomic arousal in the absence of explicit (CS) cues should be investigated. This could be easily tested using the appetitive Pavlovian discrimination paradigm, either (i) by introducing a CS-US trace interval or (ii) by measuring the decay of autonomic arousal following termination of the CS without a US, compared to continued presentation of the CS without a US ('probe' sessions as described in (Reekie et al., 2008). If sgACC/25 inactivation were to disrupt sustained

arousal in the absence of explicit external cues, then this would suggest a direct involvement of sgACC/25 in aspects of appetitive behaviour.

Uncovering the role of sgACC/25 in normal behaviour/physiology may also provide insight into whether the deficits associated with over-activation are the result of inappropriately activating a physiological system (*i.e.* 'normal' physiological function at an inappropriate time) – or whether the deficits are associated with elevating activity to supra-physiological levels that would only otherwise be achieved in a pathological state (*i.e.* abnormal physiological function)?

7.2.2 Novel antidepressant agents

The efficacy of ketamine demonstrated herein highlights its utility in treating reward-related dysfunction relevant to psychiatric disorders. Furthermore, given its sensitivity to treatments, the anticipatory anhedonia induced by sgACC/25 over-activation constitutes a valuable preparation in testing the efficacy of antidepressants in reward-related domains – whether this be chronic administration of SSRIs, or determining the efficacy of novel agents including ketamine metabolites such as (2R,6R)-hydroxynorketamine (Zanos et al., 2016). The benefits of this preparation over the sucrose preference test lie in its distinction between anticipatory and consummatory elements of reward processing, together with its more comprehensive quantification of positive affect as measured by both autonomic and behavioural aspects.

The importance of sgACC/25 over-activity in treatment response has long been recognized: successful responses to both SSRI therapy and CBT are associated with diminished activity within sgACC/25 (Mayberg, 1997), and modulation of sgACC/25 has already been implicated in the action of ketamine (Nugent et al., 2014). Whilst structural remodelling in the vmPFC has been suggested to be responsible for the effects of ketamine over hours-days, Arnsten and colleagues cite the ultra-rapid effect of *intranasal* ketamine to suggest that rapid actions of ketamine depend on short term NMDA receptor antagonism of structures directly above the cribriform plate: namely, sgACC/25. It would be interesting to determine (i) the consequences of infusions of ketamine directly into sgACC/25 on over-activation induced impairments; and (ii) whether direct infusions of ketamine into sgACC/25 could expedite ketamine's effect to ameliorate sgACC/25 over-activation induced impairments.

7.3 Appraisal of methodological approaches

Theoretical conclusions drawn from the work presented in this thesis must be considered in the context of an equally important critical appraisal of the methodological approaches employed. The predominant manipulations utilized in this thesis were pharmacological manipulations using intracerebral microinfusions via indwelling cannulae. There are both advantages and disadvantages associated with this technique, shown in **Table 7-1**.

Advantages	Disadvantages
Localisation of function by implanting cannulae to target specific neural structures	The radius over which infused drugs spread has not been precisely characterised in marmosets – however, rodent studies do indicate that spread is restricted to single brain regions (Allen et al., 2008)
Manipulation of intact cerebral cortex in the absence of significant irreversible damage	Chronic implants are associated with inflammatory responses which can lead to localised gliosis (Hayn and Koch, 2015)
Can be used in combination with non-invasive imaging (e.g. ^{18}F-FDG PET) to investigate downstream changes in neural circuits	Pharmacological manipulations are acute, whereas dysfunctional activity associated with psychiatric disease is chronic
Administered drugs, in general, do not affect fibres of passage (unlike ablative lesions)	Pharmacological agents are not cell-type specific, affecting neurons and interneurons in all cortical layers
Fewer animals needed (no sham and lesion group) and increased statistical power as animals can act as their own controls in a within-subject design	Relatively labour intensive, requiring continual maintenance to ensure patency of guide cannulae and prevent infection

Table 7-1 Appraisal of methodological approaches. Advantages and disadvantages of pharmacological manipulations using intracerebral microinfusions via indwelling cannulae.

I firmly believe that there are several advantages of reversible pharmacological approaches over lesion techniques which cannot be gainsaid. Ablative lesions destroy fibres of passage which severely confound interpretation regarding the function of cortical regions targeted. Whilst excitotoxic lesion approaches avoid this complication (through selective destruction of cell bodies), they are nevertheless associated with chronic, compensatory brain changes which again confound interpretation.

Two of the disadvantages associated with reversible pharmacological manipulations highlighted above are worth further consideration, as they pertain to the functional interpretation of the data presented in this thesis.

First, '*pharmacological manipulations are acute, whereas dysfunctional activity associated with psychiatric disease is chronic*.' As has been evidenced, acute induction of sgACC/25 over-activity induces blunted reward processing and heightened anxiety showing face-validity

to diseased states. However, disease states are not associated with isolated, acute changes in brain function – they are associated with chronic, long-term changes in a distributed network of structures. For instance, it is a *tonic* elevation in sgACC/25 activity that has been associated with depression compared to acute changes associated with transient sadness induction in healthy controls (Holtzheimer and Mayberg, 2011). At a cellular level, sustained changes in neural activity are more likely to induce depletion of neurotransmitter at synaptic terminals, receptor desensitisation/endocytosis, neuroplastic changes (long term potentiation/depression) and structural remodelling. Whilst acute pharmacological manipulations may induce some of the short-to-intermediate-term changes (neurotransmitter depletion and receptor desensitization), the time course of action of these manipulations (minutes to hours) (Lomber, 1999) is simply not long enough to induce neuroplastic or structural alterations. At a circuit level, sustained changes in activity of a single brain region are more likely to induce compensatory in other brain regions and brain networks (themselves an emergent property of the cell-level changes) which may be critical to the disease-related phenotype. Ultimately, at a cognitive level, mental illnesses would be associated with maladaptive new learning over time, which further contributes to behavioural, subjective and executive sequelae of psychiatric disorders. Therefore, acute pharmacological manipulations have their limits in terms of informing us about the changes taking place in clinically diseased states.

Does this bring us in a full circle – back to lesions, which permanently damage brain regions? Long-term observations of cognitive, behavioural and autonomic changes following excitotoxic lesions of specific brain regions may be of use if the pathophysiological change associated with the disorder is one of chronically reduced activity (in depression: the dACC/dmPFC) (Mayberg, 1997). However, comparing phenotypes associated with total cell death *vs.* chronically reduced activity (where the brain region may still be functioning but at a reduced level) remains a problem. Furthermore, in the case of sgACC/25, the pathophysiological change is one of over-activity and is therefore not amenable to be studied using lesion techniques alone. I would propose that developing methods for the chronic administration of pharmacological agents in a brain-region specific manner is of use, unveiling the possibility of investigating both chronic increases *and* decreases in activity. One technique to induce chronic increases in activity involves targeted infusions of short inhibitory RNA (siRNA) to induce persistent deficits in the translation of – for example – glutamate reuptake transporters, thereby increasing extracellular glutamate and post-synaptic neural activity (siRNA targeting EAAT2 is being used by Francesc Artigas, where they have observed *opposite* effects to those obtained with temporary pharmacological manipulations of the same molecular target using DHK; private communication). Alternatively, *Designer Receptors Exclusively Activated by Designer Drugs* (DREADDs)-based techniques show

promise (Roth, 2016), in which receptor ligands could be chronically administered without the need for indwelling cannulae (see below).

Second, '*pharmacological agents are not cell-type specific, affecting neurons and interneurons in all cortical layers.*' A lack of cell-type specificity is an issue associated with both lesion- and pharmacological-based approaches. For example, the $GABA_A$/$GABA_B$ receptor antagonists muscimol/baclofen are likely to inactivate *all* neurons in their radius of spread, since these receptors are found on the cell membranes of glutamatergic pyramidal neurons and GABAergic interneurons alike. In theory, the resultant effect of muscimol/baclofen infusions into a brain region would therefore be a function of the pre-manipulation balance of excitation and inhibition ('turning off' a dominant inhibitory tone could theoretically result in increased output). In reality, the volume and concentration of muscimol/baclofen administered likely induces complete silencing of all neurons in the radius of spread (Higgs et al., 2014).

To over-activate sgACC/25, the drug used most extensively in this thesis is DHK – an EAAT2 glutamate reuptake transporter inhibitor. By virtue of its target, the action of DHK is to some extent cell-type specific: EAAT2 is overwhelmingly expressed on astrocytes, glial cells responsible for >90% of glutamate reuptake in the CNS (Bar-Peled et al., 1997; Cisneros and Ghorpade, 2014). Application of DHK would therefore elevate levels of glutamate in the synaptic cleft. However, even though the molecular target of DHK is cell-type specific, the consequences of elevated synaptic glutamate will *not* be specific to a class of neuron. The issue remains: whilst pharmacological manipulations are region-specific and cell-body specific, they are not cell-type specific.

DREADDs-based techniques – mentioned above – can overcome issues of cell-type specificity and can be used to induce chronic elevation/depression of activity in a regionally-specific manner. In a one-off surgical procedure, a safe virus (typically adeno-associated virus, AAV) is infused into the brain region of interest. The virus contains the genetic information necessary for the expression of a protein-engineered G-protein coupled receptor (GPCR) which can be excitatory (G_q/G_s-coupled) or inhibitory (G_i coupled). The expression of this receptor can be coupled to a specific promoter such as CaMKIIa, which is thought to be relatively specific to layer V pyramidal output neurons. These receptors are not active until a ligand – typically the metabolite of clozapine, clozapine-*N*-oxide (CNO) – is administered either peripherally or centrally. The ligand, which itself purportedly lacks physiological efficacy in isolation (although see e.g. (MacLaren et al., 2016)), acts via the DREADD GPCR to induce excitatory or inhibitory changes in specific cell populations. CNO is typically administered peripherally, and can be given acutely via injection, or can be delivered via a subcutaneous osmotic mini-pump (or in drinking water) to mimic chronic alterations in activity

(e.g. in (Donato et al., 2017)). In preliminary work (not presented in this thesis), we have infused a CaMKIIa-coupled G_q DREADDs construct into sgACC/25 (Subjects 9, 17 and 18 of cohort 3 described in **TABLE 2-3**) and found that acute subcutaneous administration of 10mg/kg CNO elevates anxiety on the HI test and impairs arousal responses to an appetitive CS+ without affecting reward consumption. These results are the same as those obtained from pharmacological over-activation using DHK. DREADDs-based techniques represent a promising approach for targeted manipulations of specific cell types in specific brain regions, over short- and long-term periods.

7.4 FUTURE DIRECTIONS

The work described in this thesis lays the foundation for several avenues of future work. With reference to the proposals outlined in **FIGURE 7-5**:

A. Novel, DREADDs-based techniques could be used to activate specific neuronal populations within sgACC/25 – in particular, layer V pyramidal output neurons using the CaMKIIa promoter. DREADDs techniques could further be used to investigate the importance of efferent pathways originating from sgACC/25 in the over-activation induced phenotype. For example, by infusing DREADDs virus into sgACC/25 and cannulating the insula, amygdala, BNST or nucleus accumbens, CNO could be infused into these target structures and trigger region-specific terminal neurotransmitter release. This would test the causal contributions of specific pathways in, for example, enhanced contextual anxiety *vs.* reduced reward anticipation/motivation.

B. The effects of chronic sgACC/25 over-activity could be investigated using siRNA targeting EAAT2 (thereby directly comparing against acute EAAT2 blockade using DHK); or alternatively with chronic administration of CNO (through water or via osmotic minipumps) in animals with G_q-coupled DREADDs targeting sgACC/25.

C. What are the underlying changes responsible for enhanced arousal in aversive contexts, but reduced cue-specific arousal in appetitive contexts? Several theories have been posited above, but they require investigation. Precisely how to investigate increased rumination is difficult, although comparing the results of ^{18}F-FDG PET imaging following sgACC/25 over-activation in both appetitive and aversive contexts may provide insight into whether similar or different neural structures are involved in the different phenotypes. Furthermore, sgACC/25-insula connectivity may underlie some of the key features of the over-activation induced phenotype, so infusions directly targeting the insula together with disconnection protocols targeting both regions may serve to address this.

In addition, further experimental work could probe the anhedonic-like effects of sgACC/25 over-activation. For instance, investigating the effects of over-activation on behavioural contrast may provide further insight into whether the effects of this manipulation are linked to reduced valuation of reward. In behavioural contrast, the magnitude of an instrumental behaviour to obtain reward is proportional to reward received on the previous trial (e.g. if a large reward is received, then the animal will work harder on the subsequent trial, with the opposite being true for a small reward). If sgACC/25 over-activation reduces the behavioural discrimination between different reward sizes, this would further support an anhedonic-like effect of the manipulation.

D. The similarity – together with distinct differences – in the pattern of changes associated with peripheral injections of cortisol to those induced with sgACC/25 over-activation suggests that further investigation into the links between sgACC/25 activity and HPA axis output is important. This work is further warranted by data presented in **Chapter 5**, suggesting that sgACC/25 may elevate HPA axis output in aversive contexts. In particular, given data from the rodent implicating vmPFC in negative feedback circuits controlling cortisol release (McKlveen et al., 2013), it would be intriguing to investigate the function of GCRs *within* sgACC/25 in relation to the HPA axis, together with their importance in broader behavioural and physiological functions. This could be achieved by infusing cortisol into sgACC/25 (to activate GCRs) or infusing mifepristone (to antagonise GCRs).

E. Elucidation of the cognitive, behavioural and physiological functions requiring an 'on-line' sgACC/25 may give further insights into the mechanisms at play when its activity is disrupted. Previous work in marmosets has shown that sgACC/25 inactivation abolishes autonomic and behavioural arousal associated with negative emotional expectation (Wallis et al., 2017) but not arousal during positive emotional expectation (**Chapter 4**), and work in macaques (using ablative lesions) has suggested that sgACC/25 may be involved in sustaining autonomic arousal in the absence of specific cues (Rudebeck et al., 2014). How these functions are linked – or, in the case of ablative lesions, whether they are functions of sgACC/25 at all – remains unclear. DREADDs based techniques using G_i-coupled constructs could again provide further insight into these questions.

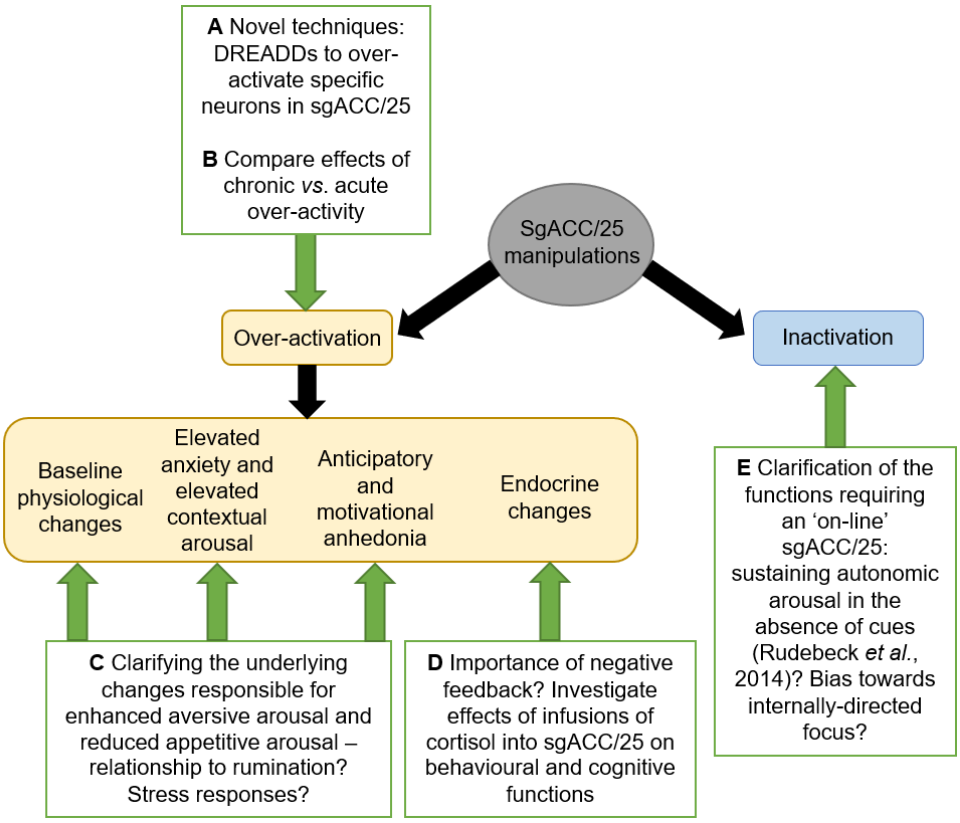


Figure 7-5 Suggestions for future work based on the data presented in this thesis. Proposals A and B concern future methodological approaches for over-activating sgACC/25; proposals C and D concern future studies to clarify the mechanistic basis of the over-activation induced phenotype; and proposal E concerns future studies probing the neurophysiological function of sgACC/25. See text for further details.

In addition to these fundamental questions regarding sgACC/25 function and dysfunction, the translationally-relevant profile of symptoms associated with sgACC/25 over-activation provides further opportunity to test the effects of existing and novel antidepressant compounds and uncover more detail about their mechanism of action. Furthermore, to supplement and support this extensive body of functional work, the continuation of retrograde and anterograde anatomical tracing studies will provide evidence for connectivity that may be affected by, and mediate the consequences of, sgACC/25 manipulations.

7.5 Conclusion

Taken together, the results described in this thesis identify the major, causal contributions of sgACC/25 over-activity to physiological and behavioural changes that typify symptoms of anhedonia, enhanced negative affect and constitute key features of major psychiatric disorders such as depression and anxiety. These impairments appear to be differentially sensitive to treatment with the novel antidepressant ketamine. Current and future work using ^{18}F-FDG PET imaging will compare the network-wide changes following sgACC/25 over-activation in both appetitive and aversive contexts, and the modulation of these circuits by ketamine to investigate its potential differential efficacy in specific symptom domains.

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